Molecular Pathogenesis of Osteonecrosis of the Femoral Head

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Abstract (In English)

Molecular Pathogenesis of Osteonecrosis of the Femoral Head

Avascular necrosis of the femoral head (ANFH) is manifested terminally by death of bone cells that results in impairment of normal reparative processes along microfractures in the femoral head. The understanding of its pathophysiology and its progression is important as we are currently unable to predict who will develop ANFH. Amongst several pathogenic mechanisms, the *vascular hypothesis* is the most compelling in which, decrease in local blood flow in the femoral head has a pivotal role in the pathogenesis of ANFH.

Dysregulation of endothelial cell (EC) activating factors and stimulators of angiogenesis, EC damage, EC apoptosis, regional endothelium dysfunction and thrombophilia, particularly impaired fibrinolysis, could lead to microvascular thrombosis important in *vascular hypothesis.*

Although ANFH pathogenesis is presumed to involve multiple hits, it is well known that ANFH tends to develop after exposure to glucocorticoids (GC), one of the major risk factors for this condition. It is known that GC have direct and indirect effects on cells. The direct effects, in particularly apoptosis of osteoblast and osteocyte, appears in our studies to play a predominant role in the pathogenesis of ANFH.

We characterized an inbred rat strain (Wistar Kyoto) susceptible to develop steroid-induced osteonecrosis. We tested the hypothesis that GC contribute to a differential gene expression in the femoral head of rats with early ANFH versus placebo-treated rats. Rat exon array, confirmed by real time PCR amplification, demonstrated a significant upregulation of a number of specific genes, particularly alpha-2-macroglobulin. Besides, several genes involved in the dynamic remodeling structure of the femoral head were differentially expressed suggesting that if the balance between degradation and repair (bone remodeling) became shifted to degradation and bone loss, a failure of structural integrity at the subchondral region could occur.

Together, our findings suggested that glucocorticoid-induced ANFH in rats might potentially be mediated at least in part, by a specific gene sharing hemostatic, osteogenic and cartilage properties.

Abstract (In French)

Pathogénie Moléculaire de l'Ostéonécrose de la Tête de Fémur

La nécrose avasculaire de la tête fémorale (NATF) est manifestée terminalement par la mort des cellules osseuses ce qui a pour résultat la diminution du processus réparatif le long de microfractures dans la tête fémorale. Une meilleure compréhension de la pathophysiologie et de la progression de la NATF permettrait éventuellement de prévoir ou prévenir ses complications parfois désastreuses. Parmi plusieurs mécanismes pathogéniques, l'hypothèse la plus fréquemment mise de l'avance est une origine vasculaire par laquelle une diminution du flux sanguin local dans la tête fémorale aurait un rôle crucial dans la pathogénie de NATF. En accord avec cette hypothèse vasculaire, la dysrégulation des facteurs d'activation des cellules endothéliales (CEs) et des stimulateurs de l'angiogénèse, des dommages aux CEs, leur apoptose, et une dysfonction régionale de l'endothélium et de facteurs thrombophiliques, en particulier des altérations de la fibrinolyse pourraient mener à la thrombose microvasculaire importante observée dans la NATF. Bien que la pathogénie de la NATF implique vraisemblablement de multiples étapes, on sait que la NATF a tendance à se développer après l'exposition aux glucocorticoïdes (GC), un des facteurs de risque majeur pour cette condition. Il est bien connu que les GCs ont des effets directs et indirects sur les cellules. Les effets directs, dont notamment l'apoptose des ostéoblastes et des ostéocytes, semblent selon nos études jouer un rôle prédominant dans la pathogénie de la NATF. Nous avons caractérisé une souche autogame (*inbred*) de rats (Wistar Kyoto) susceptibles au développement de l'ostéonécrose induite par les stéroïdes. Nous avons évalué l'hypothèse que les GCs contribuent à une expression différentielle de gènes dans la tête fémorale des rats avec NATF précoce, en comparant les rats Wistar Kyoto traités avec des stéroïdes qui ont développé la NATF avec des rats ayant recu un placebo. L'analyse de jeux ordonnés de microéchantillons d'exons de rat, confirmé par la réaction de polymérase en chaîne (RPC) en temps réel, a démontré une surexpression de gènes spécifiques, en particulier

l’alpha-2-macroglobuline. De plus, plusieurs gènes impliqués dans la structure et le remodelage dynamique de la tête fémorale ont été différentiellement exprimés suggérant qu’un déséquilibre entre la dégradation et la réparation de l’os entraîne la dégradation, la perte d’os, ainsi qu’une perte d’intégrité structurale à la région souschondrale de la tête fémorale. Dans l’ensemble, nos observations ont suggéré que la NAFT induite par les glucocorticoïdes chez les rats pourrait être la conséquence, du moins en partie, de la surexpression d’un gène spécifique en l’occurence l’alpha-2-macroglobuline qui s’approprie à la fois de propriété hémostatique, ostéogénique et découlant du cartilage.

Introduction

Avascular necrosis of the femoral head (ANFH) is manifested terminally by death of bone cells that results in impairment of normal reparative processes along microfractures in the femoral head. Besides the femoral head, which is the most common area for avascular necrosis (AVN), AVN can occur in other bones such as talus, scaphoid (21), humerus (14) and mandible (26), which like the femoral head have a terminal circulation. This disease is a disabling and progressive condition, which, if untreated, leads to femoral head collapse requiring total hip replacement. Approximately 5% to 18% of the 500,000 total hip replacement procedures performed annually in the United States are related to ANFH (2). Although the actual prevalence of the disease is unkown, an estimated 10,000 to 20,000 new patients with ANFH are diagnosed each year in the United States (1) and three hundred thousand to six hundred thousand people have the disease there. Almost 75% of patients with ANFH are between 30 to 60 years of age (2). Since most patients with the disease are at the peak of their productive years, there is a considerable effect on the workforce and direct economic consequences. Furthermore, multiple studies have reported high failure rates in total hip replacements in short-term and mid-term follow up and thus the patients have to suffer from additional surgeries (13).

As hip-preserving therapies are most effective in the earliest stages of ANFH, the key to successful treatment lies in identifying the population at risk and quantifying their risk in terms of clinical and pathophysiological characteristics so that early diagnosis can be made before femoral head collapse. Although many pathophysiologic mechanisms for ANFH have been proposed so far, critical gaps in our understanding of the pathogenesis of this disorder have direct bearing upon our ability to intervene therapeutically for hip preservation. Amongst several pathogenic mechanisms, the vascular hypothesis is the most compelling in which decrease in local blood flow in the femoral head has a pivotal role in the pathogenesis of ANFH. Endothelial cell damage could be followed by thrombus formation, with resulting degeneration distal to the site of vascular occlusion. Based on the crucial roles of the endothelium in thrombus formation, we postulated that potential defects at the level of the endothelium may contribute to the risk of developing

ANFH. The study of the pathogenesis of ANFH has two major limitations: the unavailability of longitudinal studies in humans and the lack of suitable animal models. Developing a suitable animal model that may allow identifying genes contributing to the risk of ANFH and plausibly in humans, would be very important. One goal of our study was to identify a strain of inbred rats that might be susceptible to steroid-induced ANFH as steroids are the second most important risk factor after trauma in ANFH (2). Comparison of gene expression, particularly in endothelial cells between the ANFH models and their controls may allow us to identify “risk” or “modulator” genes. In our study, the rationale for using rats instead of a mouse model had to do primarily with the size of the femoral head. The very small diameter of mouse femoral head (1-2 mm) would have made it very difficult to obtain tissue sections and sufficient material for analysis. Steroid-induced ANFH has been described in mature Japonese white rabbits (Kbs-JW) (27) but the genome of rabbit has only been incompletely sequenced, thus limiting the usefulness of that model for the identification of genes affecting the risk of developing ANFH. ANFH has also been described in spontaneously hypertensive Wistar rats (31), but the relevance of this model to ANFH occurring in humans is unclear as there is no known association between hypertension and ANFH in humans. Studying the gene profile of cells located at the femoral head especially endothelial cells will likely facilitate the identification of genes contributing to the risk of ANFH, and this information would be extremely valuable.

Review of literature

Nomenclature: The name avascular necrosis (also known as osteonecrosis [ON], aseptic, or ischemic necrosis) may be misleading, as it has not been demonstrated that bone cells die by necrosis. Indeed, the cell swelling and inflammatory responses that characterize necrosis in soft tissues do not occur (39).

*Etiology***:** Clinically, osteonecrosis of the femoral head is closely associated with several factors, such as chronic alcoholism, smoking, sickle cell disease, decompression sickness, and corticosteroid therapy for either collagen disease or renal and cardiac transplantation. These conditions are thought to be associated with increased intraosseous pressure, intravascular coagulation, or fat embolism in the bone (13). Osteonecrosis of the femoral head may be idiopathic or secondary to numerous diseases listed in table 1 (chapter 1).

*Pathogenesis***:** The pathogenesis of ANFH appears to be multifactorial (2) and three main pathways have been described: (1) a direct effect on bone with apoptosis of osteocytes and osteoblasts; (2) stimulation of intra-medullary lipocyte proliferation and hypertrophy resulting in reduced blood flow; and (3) an effect on vascular endothelial and smooth muscle cells with further stasis and ischemia (22).

*Clinical features***:** The clinical manifestations of osteonecrosis are very variable. It is often multi-articular and commonly bilateral, especially when associated with the use of glucocorticoids. Weight bearing joints (hips and knees) are most frequently affected, but involvement of other joints such as ankles has also been reported. In some cases the disorder is asymptomatic and spontaneous resolution may occur, particulary with small lesions. This phenomenon has also been observed in animals (10). In the majority of patients there is limping, pain and limitation of movement with progression of the disorder to eventual collapse of the affected joints (3).

*Diagnosis***:** Although several radiological classifications of ANFH have been described, lesions may be under-recognized on plain radiographs. Magnetic resonance imaging has been shown to have as much as 99% sensitivity for detections (3).

*Gender***:** The results vary between the different studies (3), but overall with the exception of systemic lupus erythematosis (SLE) patients, the disease is seen predominantly in males (7:3 male-female ratio) (2).

*Treatment***:** Decisions on treatment are difficult because of the variable natural history of the disease. While it appears appropriate to observe and monitor asymptomatic patients who have small lesions, some interventions are warranted for those with more extensive disease and associated with pain. The use of analgesic drugs, discontinuation of physical activity and avoiding weight-bearing are frequently advised. Core decompression, arthrodesis and joint replacement are among the possible surgical procedures. External electrical stimulation/capacitance coupling and the use of hyperbaric oxygen, which is of no clear benefit, represent possible alternatives (19,25).

Treatment in future: Until recently, core decompression of the affected osseous area was the standard approach but autologous mesenchymal stem cells (MSCs) transplantation could potentially complement ANFH treatment by adding fresh “osteogenic cells” to the healing process (38).

Besides, anti-coagulant therapy could be used for the prevention of steroid-induced ANFH in SLE (29).

Recent studies have shown that lovastatin, which increases *bone morphogenetic protein-2* (BMP-2) gene expression in rodents, could reverse the effects of glucocorticoids on bone, and prevent glucocorticoid-induced osteonecrosis in chickens and humans, considered as a potential treatment for glucocorticoid-induced osteonecrosis (8).

Chapter 1

A number of studies have examined the pathogenic mechanisms of osteonecrosis in the femoral head and none of the current hypotheses has been firmly established, although some appear more plausible than others. Furthermore, several factors may act in combination to produce ANFH. The most widely accepted central mechanism is ischemia, which may be related to direct blood vessel injury (posttraumatic necrosis), intraluminal obliteration (vasculopathy), or extraluminal obliteration in bone marrow (2).

Recently other mechanisms such as direct toxic effects on bone marrow and bone cells (17), apoptosis of bone cells (39), impairment of healing process and inhibition of angiogenesis (35,39) has been postulated.

Amongst the different hypotheses, the vascular hypothesis presents the most widely accepted etiological mechanism of this condition (2). Since endothelium plays a key role in thrombus formation, we postulated that potential defects at the level of the endothelium located at the femoral head may contribute to the development of ANFH. In the following publication, we describe the vascular hypothesis and the plausible role of endothelium in the pathogenesis of ANFH in details.

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Avascular Necrosis of the Femoral Head: Vascular Hypotheses

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Abstract

Vascular hypotheses provide compelling pathogenic mechanisms for the etiology of avascular necrosis of the femoral head (ANFH). A decrease in local blood flow of the femoral head has been postulated to be the cause of the disease. Several studies in human and animal models of ANFH have shown microvascular thrombosis. Endothelial cell damage could be followed by abnormal blood coagulation and thrombus formation with any resulting degeneration distal to the site of vascular occlusion. Other studies suggest that thrombophilia, particularly impaired fibrinolysis, plays a potential role in thrombus formation in ANFH.

Reduction in shear stress due to decreased blood flow could lead to apoptosis of endothelial cells, which can ultimately contribute to plaque erosion and thrombus formation. Dysregulation of endothelial cell activating factors and stimulators of angiogenesis or repair processes could also affect the progression and outcome of ANFH. Likewise, regional endothelium dysfunction (RED), referred to as a potential defect in endothelial cells located in the feeding vessels of the femoral head itself, may also have a crucial role in the pathogenesis of the ANFH. Molecular gene analysis of regional endothelial cells could also help to determine potential pathways important in the pathogenesis of ANFH.

Coronary Disease of the Hip

Avascular necrosis of the femoral head (ANFH) is manifested terminally by death of bone cells that results in impairment of normal reparative processes along microfractures in the femoral head. This can lead to eventual collapse of the architectural bony structure of the femoral head leading to joint pain and loss of function (Assouline-Dayan et al., 2002). As early as 1934, Phemister proposed that vascular abnormalities resulted in thrombosis and embolism contributing to the development of ANFH (Jones et al., 2003). In 1949, Chandler first compared ANFH with myocardial infarction. He

proposed that the anatomy of the head of the femur and the heart are similar. The heart was seen as protruding into its pericardial sack much in the same manner the head of the femur protrudes into the synovial capsule of the hip joint. The nutrient vessels of each area are tortuous and have limited anastomoses. Impairment of circulation, due to vascular obstruction by any means, is followed by degenerative changes in either myocardium or bone. The degree of degeneration and the effectiveness of repair depend on the extent of vascular impairment and establishment of an adequate collateral circulation (Chandler, 2001). Like the heart, the vascular supply to the femoral head is affected by many endogenous and exogenous factors (Table 1) (Jones, Jr., 1999). Common belief is that all of these factors run through diverse pathways to focal thrombosis and eventual ANFH (Jones, Jr., 1999).

Glucocorticoids are the most common non-traumatic cause of ANFH (Assouline-Dayan et al., 2002). Between 5%-40% of patients treated with long term glucocorticoids develop ANFH (Koo et al., 2002). Obviously not every patient treated with high dose steroids develops ANFH. Likewise, although alcoholism is associated with ANFH as an etiological factor, not all heavy alcohol users develop the disease. Therefore, it is difficult to say whether ANFH that occurs in patients with an underlying or predisposing factor, is actually due to the underlying disease or risk factor rather than the glucocorticoids or alcohol. Many of the patients with these predisposing factors or underlying diseases such as systemic lupus erythematous or acute leukemia also have been administered glucocorticoids as part of their treatment regimen. As well, several investigators have reported patients developing ANFH before taking glucocorticoid or with a replacement treatment regimen (Khan et al., 2001; Freeman and Kwan, 1993; Scribner et al., 2000; Koller et al., 2000). Likewise, while most therapeutic regimens in oncology include steroids as part of the protocol or as an adjunctive measure in the control of nausea and/or prophylaxis against hypersensitivity reactions, cases of ANFH developing after chemotherapy (with or without steroids) or radiation therapy have been well described in the oncologic literature (Talamo et al., 2005; Dawson et al., 2001; Macdonald and Bissett, 2001). The role of GVHD and/or its treatment has also been incriminated (Enright et al., 1990). Certainly the current pathophysiologic model of ANFH puts forward a multiple hit theory in which with increasing number of risk factors, the risk of ANFH increases (Table

1) (Schulte and Beelen, 2004). Although many pathophysiological mechanisms for ANFH have been proposed, critical gaps in the understanding of the pathogenesis of ANFH still persist. Amongst several confounding pathogenic mechanisms for ANFH, a vascular hypothesis appears to be the most compelling, presuming that decrease in local blood flow in the femoral head has a pivotal role in the pathogenesis of ANFH (Figure 1) (de Camargo et al., 1984).

Histological studies of Hirano et al showed severe luminal stenosis of the draining vein at the outlet portion of the femoral head and in the extraosseous area in the early stages of ANFH (Hirano et al., 1997). In another study, Starklint et al found a widespread obstruction in the blood flow of the femoral head in late stage of avascular necrosis. The venous outflow was further impaired by thrombi and by perivenous concentric fibrosis, which considerably reduced the lumen of the veins and venules (Starklint et al., 1995). Several investigators have shown elevated intrafemoral pressure in ANFH (Hungerford and Lennox, 1985; Pedersen et al., 1989; Ho et al., 1988). As the intraosseous pressure rises, sinusoidal compression causes venous stasis, and eventually arterial obstruction occurs, accounting for ischemic osteonecrosis. Osteonecrosis does not always happen in the presence of circulatory disturbance caused by intraosseous pressure (Atsumi and Kuroki, 1992) and the outcome of elevated intraosseous pressure depends to a large extent on additional parameters. Welch et al experimentally produced intraosseous hypertension and demonstrated endosteal, periosteal and cancellous new bone formation, but paradoxically no osteonecrosis (Welch et al., 1993). Elevated intraosseous pressure may also be seen in osteoarthritis (Ho et al., 1988; Arnoldi et al., 1975) and therefore, is not specific to ANFH.

Angiography studies of hips in early stage of ANFH, before gross radiographic changes, revealed the absence of superior retinacular arteries, which are the most important arteries feeding the proximal and weight bearing areas of the femoral head. At this stage, revascularization with limited arterial penetrations has been observed (Atsumi and Kuroki, 1992). Atsumi et al showed, concurrently with radiological evidence of necrosis, a deeper peripheral arterial penetration with the formation of vessels of increasingly greater diameter in the absence of the real superior retinacular arteries. Although revascularization improved angiographic findings, the collapse of the femoral

head proceeded further. They hypothesized that interruption of revascularization in affected hips occurred because of microscopic stress fractures and eventual subchondral fracture line propogation during the repair process (Atsumi et al., 1996). After revascularization naturally occurred, the reparative process itself results in bone turnover with osteoclast activation and lay down of weaker woven bone. Collapse could also be explained by the fact that in subchondral bone, bone formation occurs at a slower rate than resorption, resulting in the net removal of bone, loss of structural integrity, and subchondral fracture (Aaron and Ciombor, 2001). In some cases, spontaneous resolution of ANFH has been observed in early to middle stages of the disease (Cheng et al., 2004). The factors involved in establishing the disease in some patients and the factors, which lead to resolution of the disease in others are still elusive. Amongst the plausible mechanisms, “regional endothelium dysfunction (RED)” may play a pivotal role in the progression of the disease.

Table 1- Factors potentially activating intravascular coagulation and causing osteonecrosis

Familial Thrombophilia
- APC-resistance
- Protein C deficiency
- Protein S deficiency
- Antithrombin III deficiency
- Hyperhomocysteinemia

Hyperlipemia & Embolic lipid
- Alcoholism
- Carbon tetrachloride poisoning
- Diabetes mellitus
- Fat emulsion therapy
- Hypercortisonism
- Hyperlipemia (Types II and IV)
- C-reactive protein increased
- Obesity
- Pregnancy (fatty liver)
- Disrupted adipocytes
  - Dysbaric phenomena
  - Hemaglobinopathies
  - Pancreatitis (lipase)
  - Severe burns
  - Unrelated fractures

Hypersensitivity Reactions
- Allograft organ rejection
  - Kidney, heart, liver, marrow
- Anaphylactic Shock

- Antiphospholipid antibodies
- Immune complexes
- Immune globulin therapy
- Serum sickness
- Systemic lupus erythematous
- Anticardiolipin antibodies
- Lupus anticoagulant

Hypofibrinolysis
- Dysfibrinogenemia
- Plasminogen deficiency
- TPA decreased
- PAI-1 increased

Infections
- Bacterial endotoxic reactions
  - Neisseria meningitidis
  - Haemophilus influenzae
  - Escherichia coli
  - others
- Bacterial lipopolysaccharides
- Bacterial mucopolysaccharides
- Corticosteroid suppression
  - Prepares Shwartzman
- Intravenous drug abuse
- Septic abortion
- Toxic shock
  - Staphylococcus exotoxin

Viruses
- Cytomegalovirus
- Hepatistis
- Human Immunodeficiency
- Rubella
- Others

Proteolytic Enzymes
- Pancreatitis (trypsin)
- Snake Venom

Tissue Factor Release
- Inflammatory bowel disease
  - Crohn`s disease
  - Ulcerative colitis
- Malignancies
  - Acute leukemias
  - Hodgkin`s disease
  - Metastatic carcinoma
  - Pancreatitic carcinoma

- Others
- Chemotherapy
- Neurodamage
  - Brain injury / surgery
  - Spinal injury / Surgery
- Pregnancy
  - Amniotic fluid embolism
  - Fatty liver of pregnancy

Normal pregnancy
- Prepares Shwartzman
- Retained fetus in utero
- Toxemia
  - Preeclampsia
  - Eclampsia

Other Prethrombotic Conditions
- Acidosis
- Anorexia nervosa
- Anovulatory agents (estrogens)
- Cigarette smoking
- Decompression sickness
- Diabetic angiopathy
- Gaucher crisis
- Hemolytic-uremic syndrome
- Hemolysis
- Hepatic failure
- Hyperfibrinogenemia
- Hypertension
- Hypertrophy fatty marrow
- Hyperviscosity
- Hypotension (shock)
- Immobilization
- Lipoprotein (a)
- Nephrotic syndrome
- Patent foramen ovale
- Polycythemia
- Postoperative states
- Raynaud`s phenomenon
- Sickle-cell crisis
- Storage diseases
  - Fabry-Anderson disease
  - Gaucher disease
  - Polyvinylpyrrolidone (PVP)

- Thrombocytosis
- Thrombocytopenia purpura
- Vasoconstriction
- Vascular disorders
  - Aneurysms
  - Arteriosclerosis
  - Giant hemangiomas
  - Vasculitis

J. P. Jones, Acta Orthopadica Belgica, 1999, 65: 5-8

Endothelium Dysfunction and Thrombosis

The endothelial cell monolayer constitutes the inner lining of the vascular wall and plays an essential role in the homeostasis of the blood vessel. Due to its unique localization, the endothelium is continuously exposed to inflammatory cells and circulating factors which could induce endothelial activation and/or endothelial injury (Vadasz et al., 2004). The concept of a focal nature of a systemic hematological defect resulting in local hypercoagulable states has been discussed. Deficiencies of antithrombin III, protein C and protein S result in deep venous thrombosis of the extremities. Thrombotic thrombocytopenia purpura and the hemolyticuremic syndrome result in microthrombotic lesions that are detectable in all organs except the liver and lungs. Antiphospholipid-antibody syndrome results in clotting of only particular venous and arterial areas including the retina and the placenta. The heterogeneity of the endothelial cell structure amongst these many different organs has been postulated to be a probable cause for the varied clotting responses in the separate endothelial beds (Rosenberg and Aird, 1999). Regional endothelial beds (REBs) have a different local expression of systemic defects. This regional endothelium dysfunction may be a reason for ANFH.

In a study by Jacobs et al, damage or abnormality of the reticular vessels was suggested to be the underlying mechanism of ANFH (Jacobs, 1978). Damage of endothelial cells may result in abnormal blood coagulation and thrombi formation. ANFH could occur distal to the site of arterial occlusion. Slichter et al showed endothelial cell damage followed by platelet thrombus formation with secondary fibrin deposition in the femoral head in dysbaric osteonecrosis (Slichter et al., 1981). Li et al also showed endothelial cell damage as well as a high coagulant and a low fibrinolytic milieu- the possible pathologic mechanisms of glucocorticoid-induced ANFH (Li et al., 2004). Intravascular coagulation is itself an intermediary event that has both hereditary and acquired risk factors. Glueck et al suggested that thrombophilia, particularly impaired fibrinolysis, occurs in ANFH patients at higher frequency than in the general population (Glueck et al., 1994; Glueck et al., 1999). They described high levels of PAI-1 in patients with idiopathic ANFH and ascribed a role to the PAI-1 genotype in association with

ANFH (Glueck et al., 1999). There are two forms of plasminogen activator inhibitor (PAI). PAI-2 is a thromboblastic product and PAI-1 is a serine protease inhibitor that is synthesized and released by endothelial cells in blood vessel walls. PAI-1 exerts its regulatory activity on fibrinolysis by forming complexes with tissue plasminogen activator (tPA). The tPA/PAI-1 complex does not have the ability to activate plasminogen to plasmin. Increase in PAI-1 activity suppresses the generation of plasmin resulting in hypofibrinolysis and a relative hypercoagulable state (Aaron and Ciombor, 2001). In a clinical and experimental study the activity of tPA and PAI-1 decreased and increased respectively in patients with ANFH. (Li et al., 2004). In their study, Jones et al suggested that liquid fat, thromboplastin and other vasoactive substances released from injured marrow adipocytes in ANFH affect the vascular walls (endothelial cells) and produce a hypercoagulable state through the endothelial cells(Jones, Jr. et al., 1993).

Nyska et al. suggested that vascular cell adhesion molecule-1 (VCAM-1) promotes adhesion of erythrocytes to the endothelium. Increased expression of VCAM-1, produced by different types of cells not just by endothelium, acts in a paracrine mode on the endothelial cells, promoting thrombus formation. Increased expression of VCAM-1 is largely mediated by IL-1 released from hypoxic endothelial cells (Nyska et al., 2003). 6-ketone prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), a metabolite of prostaglandin I_2 (PGI_2), is considered as a marker of endothelial cell injury (Li et al., 2004). PGI_2, mainly produced by vascular endothelial cells, strongly dilates blood vessels and inhibits platelet aggregation. In a study by He et al, the level of 6-keto-$PGF_{1\alpha}$ decreased significantly compared to controls in an ANFH rabbit model induced by endotoxin and glucocorticoid. This suggests there is vascular endothelial cell impairment in ANFH (He et al., 2004).

Spontaneously hypertensive rats (SHR) have been studied as an osteonecrosis animal model (Wada et al., 2004; Wada et al., 2004; Tomita et al., 1999; Shibahara et al., 2000). Wada et al speculated that the femoral head of SHR showed some degree of endothelial cell dysfunction. Immunohistochemistry studies were negative for endothelial nitric oxide synthetase (eNOS), which catalyzes the formation of eNO (endothelial nitric oxide) (Wada et al., 2004). eNO has several anticoagulatory actions, including dilation of vessels, prevention of platelet aggregation and inhibition of monocyte adherence to the endothelium (Wada et al., 2004). Even in other tissues the activity and expression of

eNOS is lower in SHR than other strains of rats. Chou et al used western blot analysis to show that the basal activity and expression of eNOS in aorta were significantly lower in SHR than in other strains of rats (Chou et al., 1998). An eNOS knock-out model was shown to accelerate atherosclerotic lesion formation in mice (Vadasz et al., 2004). Li et al showed that the level of the serum NO decreased in glucocorticoid-induced ANFH rabbit model. The synthesis of eNO could be influenced by glucocorticoids through three potential mechanisms; direct injury effect of glucocorticoid on endothelial cells, repressing the activity of NOS and increasing the level of blood lipid which lowers the synthesis of eNO (Li et al., 2004).

Endothelium dysfunction or activation, primarily by endothelium itself or secondarily to a stimulator, could activate the thrombosis cascade, followed by ischemia and infarction. It has been suggested by Redlich et al that the thrombosis in rats represents a cycle of events in which an initial low level activation of endothelium and/or dysfunction triggered by hemolysis and hypoxia results in additional vascular problems – thrombosis, poor blood flow, infarction, and additional hypoxia (Redlich et al., 2004). Following infarction in the femoral head, a repair process begins with the entry of blood vessels into the zone of necrosis (revascularization), bone reabsorption and formation. Several essential factors such as vascular endothelial growth factor (VEGF) act directly on endothelial cells and induce angiogenesis (Yang et al., 2003b). Dysregulation of the essential factors would effect the revascularization, and consequently the repair process. Yang et al used VEGF gene transfection to enhance the repair of ANFH in a rabbit model of ANFH disease. They observed that bone repair was augmented in the femoral head treated with VEGF gene (Yang et al., 2003a; Yang et al., 2003b). Nevertheless, other reports have not been in agreement with these studies. In addition to osteoblasts, VEGF also recruits and activates osteoclasts, involved in the remodeling process of the necrotic femoral head, which may trigger a mechanically weak bony construction. Therefore, it is possible that a molecule such as VEGF-inhibitor could be an effective therapeutic intervention in ANFH (Boss et al., 2004). Generalization between 4-legged ANFH animal models and the human disease and collapse process must be cautious. Glucocorticoids and interferon (-alpha) could also inhibit the angiogenesis probably by a mechanism

involving the reduction of the proteolytic activity with the synthesis of PAI-1 (Smith, 1997).

Medications such as glucocorticoids reinforce the vascular processes leading to thrombotic occlusion. They directly injure endothelial cells and amplify hypercoagulability (Boss and Misselevich, 2003). Glucocorticoids indirectly inhibit capillary proliferation by suppressing myofibroblastic functions. Myofibroblasts, an intrinsic constituent of all granulation tissues, produce an endothelial cell growth factor, which stimulates angiogenesis (Boss, 2004). Glucocorticoids also produce significant fat accumulation in bone marrow as a consequence of both adipocyte hypertrophy and hyperplasia, generating intraosseous hypertension (Wang et al., 1985) and in particular, increase the activity of PAI-1 (Ferrari et al., 2002) leading to a hypercoagulable state, induce apoptosis in cancellous lining cells, osteocytes (Weinstein et al., 2000) and osteoblast, inhibit osteoblastogenesis (Weinstein et al., 2002; Weinstein et al., 1998; Wang et al., 1981).

Endothelial Cell Apoptosis

Decrease in local blood flow in the femoral head is key to the development of ANFH. The maintenance of the fine balance of procoagulants and anticoagulants at the endothelial cell surface is essential to maintain normal hemostasis. Heterogeneous responses to the systemic hemostasis defects according to endothelial bed location are manifested in ANFH. However, endothelial cell apoptosis is known to promote coagulopathic changes and it is hypothesized that this process could occur at the level of the bone endothelium environment. Cessation or reduction of blood flow along capillaries could play an etiologic role in endothelial cell deletion. Disturbed blood flow may also result secondarily from alterations in the rheology of erythrocytes, such as deformation, self-aggregation, and adherence to the endothelium of the blood vessel walls (Shabat et al., 2004). Adult rabbits subjected to long-term increases in carotid blood flow (28 days) exhibited endothelial remodeling by losing the endothelial cells and keeping the endothelial cell density to nearly normal levels in the narrowed artery (Langille et al.,

1989). Azmi and O'Shea detected deletion of endothelial cells by apoptosis during regression of the vasculature of the corpus luteum, and attributed this response to reduced blood flow (Azmi and O'Shea, 1984). Blood flow itself is the most potent endogenous protective force that prevents the induction of endothelial cell apoptosis (Langille et al., 1989). The association between physical force imposed on the vessel wall by blood flow (referred to as shear stress) (Langille et al., 1989) and atherosclerotic lesion formation is clearly illustrated by analysis of different regions of the vascular tree. Atherosclerotic lesions are preferentially located at branches, curvatures and bifurcations, where separation of the blood flow streamline creates low or turbulent flow with reduced shear stress exposure to the endothelial cells. Reduction in the mechanical stimulation of endothelial cells induced by shear stress, decreases the expression of eNOS, followed by lowering the synthesis of nitric oxide within the endothelial cells, leading to apoptosis of endothelial cells. Increasing evidence suggests that endothelial cell apoptosis may contribute to plaque erosion, which consequently leads to thrombus formation by two mechanisms. First, the microparticles, composed of residual bodies of apoptotic cells, can directly induce endothelial dysfunction and activate the thrombosis cascade and second, the apoptotic endothelial cells can act as a procoagulant. Apoptosis induction in endothelial cells stimulates the binding of thrombocytes to the endothelium and further induces activation of platelets, which contributes to further thrombus formation (Dimmeler et al., 2002).

Conclusion

It is now generally felt that vascular thrombosis is the major pathogenic event leading to osteocyte necrosis and eventually collapse of the femoral head in ANFH. It can be strongly argued that vascular thrombosis is complex and multifactorial. Since endothelium has an essential role in blood coagulation and preliminary investigations have elucidated an endothelium damage or dysfunction in ANFH, further studies focusing on the role of endothelium in the pathogenesis of ANFH are needed. We envision that, molecular gene analysis of endothelial cells located at the femoral head may lead to

discover potential pathways important in the pathogenesis of the disease. While more effort is needed to comprehend better the role of endothelium, trial therapy involving antithrombotic drugs that interfere with the function of endothelial cells or medical interventions promoting angiogenesis may be useful in the treatment of the patients and in future could revolutionize the treatment of ANFH.

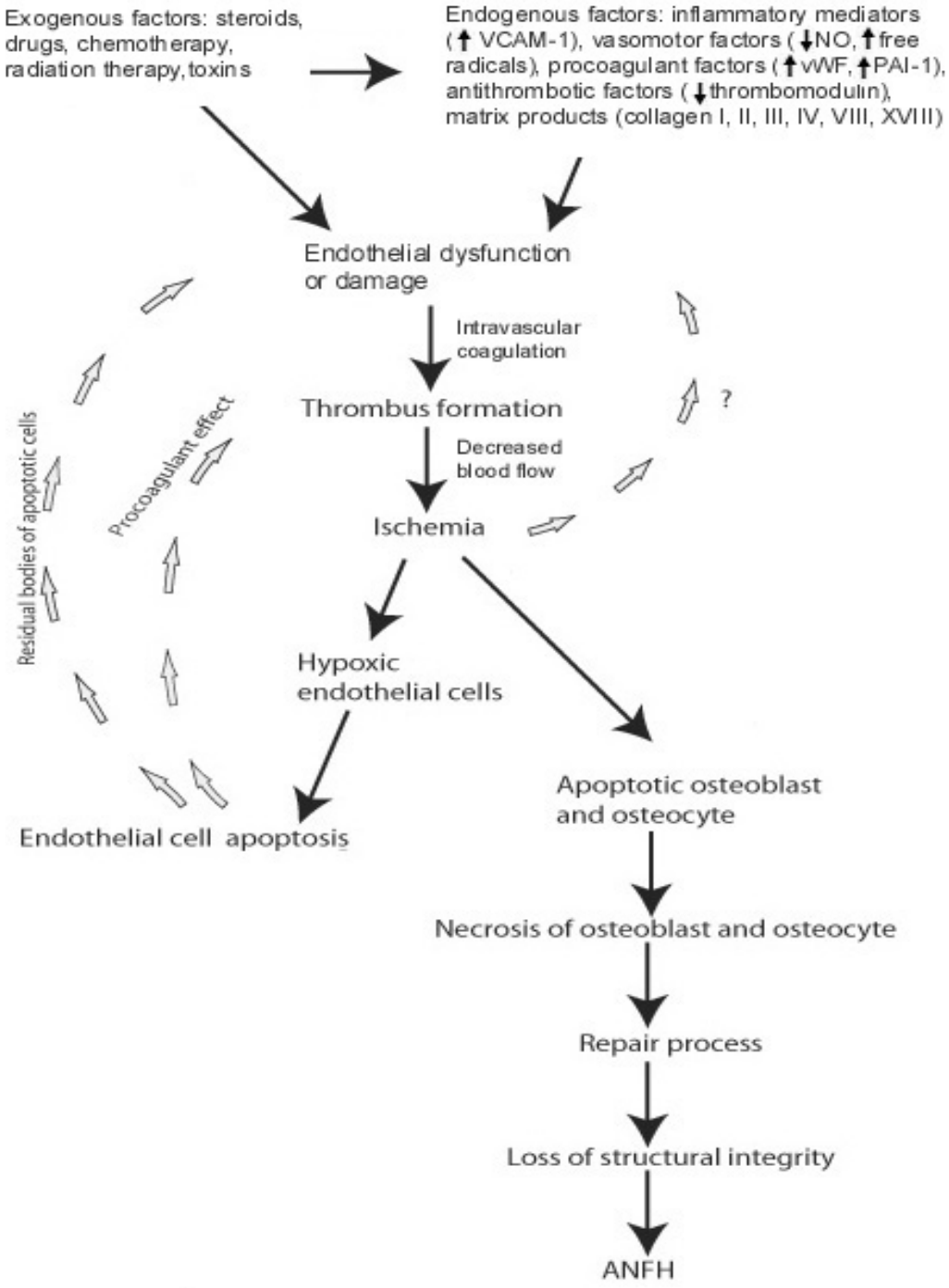

Figure 1. Schematic diagram of the vascular hypothesis in the pathogenesis of avascular necrosis of the femoral head (ANFH).

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Amendments:

It is recommended to use µmol/L and nmol/L instead of µM and nM, respectively.

Chapter 2

Steroid use has been associated with osteonecrosis, but a causal relationship is not always directly found. The first association between osteonecrosis and steroids was observed in renal transplant patients who were treated with steroids as part of their immunosuppressive regimen (16). In cross-sectional studies, 10% to 30% of the cases of osteonecrosis have been associated with steroid administration (22). However, the few prospective longitudinal studies that can be found in the literature have indicated that ostonecrosis may occur in only 8% to 10% of patients received steroid therapy (22). Thus, the incidence of osteonecrosis when steroids are the only triggering factor present is low and it is postulated that the pathogenesis of osteonecrosis is a multiple hit phenomenon. However, in patients with systemic illnesses, primarily those involving immune complex deposition, steroid use dramatically increases the incidence of osteonecrosis (2,39). The risk of osteonecrosis associated with steroids is also high in patients underlying renal transplantation (22). It is difficult to separate the effects of steroids on bone from those of the underlying diseases, such as mineralization defects and osteoporosis seen in renal and liver failure and vasculitis associated with systemic lupus erythematosus. Besides the underlying disease, the dose of steroids is an important factor to induce osteonecrosis as the incidence of osteonecrosis increases with higher dose of steroid (22).

Some believe that steroid-related osteonecrosis is the only sub-type related to apoptosis, in contrast to the blood flow deprivation-induced tissue coagulation found in idiopathic osteonecrosis (6). In the following published review, we examine different mechanisms important in the pathogenesis of ANFH.

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Glucocorticoids in Osteonecrosis of the Femoral Head: A New Understanding of the Mechanisms of Action

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Abstract

Glucocorticoid (GC) usage is the most common non-traumatic cause of osteonecrosis of the femoral head (ON). Despite the strong association of GC with ON, the underlying mechanisms have been unclear. Investigators have proposed both direct and indirect effects of GC on cells. Indirect and direct mechanisms remain intimately related and often result in positive feedback loops to potentiate the disease processes. However, the direct effects, in particularly apoptosis, have recently been shown to be increasingly important. Suppression of osteoblast and osteoclast precursor production, increased apoptosis of osteoblasts and osteocytes, prolongation of the lifespan of osteoclasts and apoptosis of endothelial cells (EC) are all direct effects of GC usage. Elevated blood pressure through several pathways may raise the risk of clot formation. High-dose GC also decreases tissue plasminogen activator activity (t-PA) and increases plasma plasminogen activator inhibitor-1 (PAI-1) antigen levels increasing the procoagulant potential of GC. Inhibited angiogenesis, altered bone repair and nitric oxide metabolism can also result. Also, GC treatment modulates other vasoactive mediators such as endothelin-1, noradrenalin and bradykinin. Thus, GCs act as a regulator of local blood flow by modulating vascular responsiveness to vasoactive substances. Vasoconstriction induced in intraosseous femoral head arteries causes femoral head ischemia. GCs also cause ischemia through increased intraosseous pressure, which subsequently decreases the blood flow to the femoral head by apoptosis of ECs as well as elevating the level of adipogenesis and fat hypertrophy in the bone marrow.

It is difficult to predict which patients receiving a specific dose of GC will develop ON, indicating individual differences in steroid sensitivity and the potential of additional mechanisms. The textbook model of ON is a multiple hit theory in which, with a greater number of risk factors, the risk of ON increases. While more effort is needed to better comprehend the role of GC in ON, newer data on GC action upon the endothelial cell and the regional endothelial bed dysfunction theory sheds new light on particular GC mechanisms. Better understanding of GC pathomechanisms can lead to better treatment options.

Background

Harvey Cushing recognized the adverse effects of hypercortisolism on bone as early as 1932. Currently, more than 30 million Americans require GC drugs as part of their treatment regime. Depending on both the duration of therapy and dose, up to 50% of steroid users develop bone loss leading to fractures [1], while up to 40% of steroid users develop some degree of ON, a debilitating skeletal disorder [2]. GCs are known to be the most common non-traumatic cause of ON [3]. In 1987, Felson and Anderson performed a meta–analysis demonstrating that mean daily GC dose was strongly correlated with the disease. In this review, a 4.6-fold increase in the rate of ON was postulated for every 10 mg/day increase in mean daily oral dose of prednisone during the first six months of therapy [4]. Most series report agree that a sustained large dose of GC is required to sustain symptomatic ON [2,5]. Rare reports of ON after low dose or no GC exposure are probably related to underlying medical conditions, coagulopathic dyscrasias and genetic susceptibility [6-11]. Unfortunately, most patients with symptomatic ON of the femoral head eventually need surgery, usually total hip arthroplasty (THA), within a few years of onset [12]. Because the average age at presentation is about 33 years of age, these patients often require multiple increasingly difficult surgeries over the course of a lifetime [13]. A more complete understanding of why GC use is associated with ON would be helpful.

New research has revealed that there may be more to the disease process than an effect on bone cells alone. Endothelial cell susceptibility may actually be just as important or more pertinent to the evolution of the pathophysiology of ON in the femoral head [14]. Several mechanisms have been postulated for the role of GC-induced ON. Some investigators assume a direct effect of the drug on bone cells. Older theories have considered an indirect effect of GC via an influence on the gonads and parathyroid glands. Recently, it has been shown that secondary hyperparathyroidism and hypogonadism appear to have no major role in the pathogenesis of GC-induced ON or in the resultant fractures [1] unlike earlier thinking. Thus, direct effect of GC on bone and other cells responsible for ON is undoubtedly more important than the indirect effect.

Role in apoptosis

Some action of GC is believed to be mediated by glucocorticoid receptors (GR) [15,16]. *In vitro* studies have shown the presence of GR in human and murine osteoblasts, osteocytes and osteoclasts [17- 22]. For the first time, Silvestrini et al. showed that GR were present in cartilage (proliferative and hypertrophic zones) and in osteoblasts, ostocytes and osteoclasts of femurs of young adult rats [23]. Natural and synthetic GC bind to GR, producing major conformational changes that result in nuclear translocation or transrepression of transcription factor. This includes nuclear factor κB, which causes modification of pivotal mediators of innate and adaptive immunity [16]. Due to the ability of GC to modulate immune responses, they have been extensively used as anti-inflammatory agents. The immune response acts commonly through the Fas pathway, one of the best-characterized apoptotic pathways. Binding of FasL to FasR causes receptor oligomerization and recruitment of an adapter protein, FADD, which interacts with caspase-8, and initiates a caspase cascade which leads to apoptosis [24] in immunogenic cells. Dexamethasone (DEXA) and other GCs could also interact through the AP1 proto-oncogenes (c-Jun and c-Fos). GR and AP1 interactions have been described in GC response elements (GREs), where these two transcriptional factors locate close together [15]. There is a relatively rapid induction of apoptosis in *in-vitro* dexamethasone-treated T-lymphocytes that occurs within a few hours of exposure [25].

Despite the apoptosis effect of GC on T-lymphocytes, DEXA can promote proliferation and protect cells from apoptosis and/or necrosis in particular conditions. This effect has been seen in corneal epithelial cells, keratocytes, epithelial cells of the mammary gland, hepatocytes, and thymocytes. The GR gene response may positively or negatively regulate this paradoxical biphasic effect through different responses in different types of cells or through different dosages. For instance, DEXA would increase cell proliferation at low concentrations (below 10 μM) in some brain tumor cells and induce cellular apoptosis and/or necrosis at high concentrations (above 100 μM in brain tumor cells or 0.0001 to 0.001M in corneal endothelial cells) [15,26]. Although not clearly defined, these dual effects of GC may also be the result of cross-talk between nuclear comodulators, or interactions of transcription factors [15].

Effects on Bone

The osteoblasts and osteocytes of the femoral cortex mostly undergo apoptosis after a lengthy period of GC medication [27]. From *in-vivo* and clinical studies, abundant apoptotic osteocytes and cells lining the cancellous bone were found juxtaposed to the subtotal fracture crescent in the femurs of the patients with GC excess [28]. A similar study by Calder and his colleagues has shown widespread apoptosis of osteoblasts and osteocytes in steroid- and alcohol-induced osteonecrosis patients [29].

O'Brien and his colleagues have shown that mice harbouring osteoblast/osteocyte-specific-11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2) transgene were protected from GC-induced apoptosis of osteocytes and osteoblasts. 11ß-HSD2, a high-affinity NAD-dependent enzyme, converts biologically active GC to its inactive metabolite and subsequently the inactivated GC would fail to activate the GR [30]. Despite the prevention from GC-induced decreased bone formation, the osteoblast/osteocyte-specific-11ß-HSD2 transgene mice did not prevent the early rapid bone loss, as osteoclasts are not protected from GC [1]. Further studies have shown that the early loss of bone with GC excess is caused by a direct effect of GC on osteoclasts to extend their life span [31]. Osteoclast survival and differentiation are regulated by factors produced by stromal and osteoblastic cells. The critical factor is the receptor activator of NF-κB (RANK) ligand, a member of the tumor necrosis factor (TNF) ligand family. RANK ligand and macrophage-colony-stimulating factor are both crucial and sufficient for osteoclast differentiation in the absence of marrow stromal cells; RANK ligand also prolongs the survival of differentiated osteoclasts [1]. The quantities of TNF-α, RANK ligand and osteoprotegerin are raised in GC-treated osteoblasts and consequently, the differentiation of osteoclasts is blocked [27]. Therefore, it is not completely clear how the process of apoptosis of bone cells alone would result in ON.

Apart from the direct effect of GC, it has been shown that GC excess could affect the birth rate of bone cells. Weinstein et al. administered prednisolone to 7-month-old mice for 27 days and found decreased bone density, serum osteocalcin, and cancellous

bone area along with trabecular narrowing. These changes were accompanied by diminished bone formation and turnover and impaired osteoblastogenesis and osteoclastogenesis [32].

Therefore, it is assumed that the pathogenesis of GC–induced bone disease may at least partially be caused by suppression of osteoblast and osteoclast precursor production in the bone marrow, increased apoptosis of osteoblasts and osteocytes, and prolongation of the lifespan of osteoclasts [1].

It is also postulated that GC–induced osteocyte apoptosis disrupts the mechanosensory function of the osteocyte network, which is believed to constitute the mechanosensor and apparatus controlling repair processes in bone [33]. This may start the inexorable sequence of events leading to femoral head collapse [1]. In addition, the end result of fracture collapse may be facilitated by the co-existing osteoporosis mediated by GC-induced osteoblast apoptosis [33].

Bone morphogenic protein-2 (BMP2) gene expression also decreases after GC treatment. Individuals who were more susceptible to a GC-induced decrease in BMP2 and osteocalcin gene expression were more likely to have ON [34].

Effects on Endothelial Cells

GC has abundant effects on endothelial cells that line the sinusoids and inner layer of blood vessels in the femoral head. Experimental evidence of GC-induced hypertension indicates that raised arterial blood pressure is related to an elevated peripheral resistance [35]. GC action resulting in vasoactive mediators is discussed below. Another hypothesis that has been advanced as a mechanism for the enhanced peripheral resistance is a decrease in the number of functional microvessels or capillary rarefaction [36]. GC can directly injure endothelial cells [14] and enhance hypercoagulability [37]. In a study by Jacobs et al, damaged or abnormal reticular vessels was suggested to be the underlying mechanism of ON [38]. Damage of endothelial cells may result in abnormal blood

coagulation and thrombi formation with ON occurring distal to the site of arterial occlusion [14].

6-ketone prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), a metabolite of prostaglandin I_2 (PGI_2), is considered as a marker of endothelial cell injury [39]. PGI_2, mainly produced by vascular endothelial cells, strongly dilates blood vessels and inhibits platelet aggregation. In a study by He et al, the level of 6-keto-$PGF_{1\alpha}$ decreased significantly compared to controls in an ON rabbit model induced by endotoxin and GC. This suggests another endothelial cell impairment in ON [40], which may be GC-mediated.

It has been postulated that apoptotic endothelial cell death serves as a mechanism for the capillary rarefaction in glucocorticoid-mediated hypertension [41]. Therefore, it is plausible that GC-induced hypertension in the femoral head disturbs the blood flow in the femoral head vessels and aborts the repair process [14]. Furthermore, enhanced blood pressure in the blood vessels of the femoral head could increase thrombin, which converts fibrinogen to fibrin, forming fibrin clots. Studies on the enzymes involved in the coagulation cascade have shown that thrombin is elevated in the established phase of hypertension as a consequence of raised blood pressure [42].

Avascular necrosis and the coagulation pathway

It has been shown in both *in-vivo* and *ex-vivo* studies, that lower doses of several types of GC will initially inhibit arterial thrombosis through inhibition of platelet aggregation. At higher doses, these effects are counteracted by a significant inhibition of the fibrinolytic activity [43-47]. The latter was shown to occur as a result of decreased tissue plasminogen activator activity (t-PA) and increased plasma plasminogen activator inhibitor-1 (PAI-1) antigen levels [43,44]. Decreased fibrinolytic activity, which may be a consequence of increased PAI-1, has been described in patients with ON [48]. In a study performed by Yamamoto et al., DEXA upregulated PAI-1 gene expression in human umbilical vein endothelial cells (HUVEC) stimulated by TNF-α conditioning. They

postulated that such inflammatory conditions (DEXA exposure and TNF-α conditioning) could promote blood procoagulant effect by acting on vascular endothelial cells [49]. Furthermore, Drescher et al. showed in an ON animal model, that plasma fibrinogen was significantly increased at the early stage of ON following a megadose steroid treatment [50] suggesting a pro-inflammatory condition in steroid-induced ON.

One could argue that GC may potentially be responsible for a systemic response resulting in ON. Some cases have shown that clinically significant ON in several joints can result from the use of high dose GC [36,51,52]. However, this is the exception rather than the normal presentation. Recently, the literature has been at odds for an opinion of whether systemic defects in the clotting mechanism actually account for clinically significant ON. Several studies have revealed that the prevalence of thrombophilic and hypofibrinolytic coagulation abnormalities in patients with GC-induced ON is increased compared to controls [9,10,53-55]. A few studies have reported that there were no significant differences in the levels of thrombotic and fibrinolytic factors [56,57]. The general irreproducibility of systemic blood clotting defects amongst patients with ON is concerning. Dozens of different blood thrombophilic factors probably cannot be responsible for a single disease process. Also, still unexplained are the many patients without known systemic defects. A mechanism by which ON of the femoral head is dependant on a local dysregulation of coagulation at the level of the femoral head can explain the disease process [14]. Clotting at the microcirculation level is more likely dependant on the local balance of pro- and hypo- coagulant factors at the endothelial cell surface of the bone regional vascular bed than it is upon the systemic clotting factors.

Slichter et al showed endothelial cell damage followed by platelet thrombus formation with secondary fibrin deposition in the femoral head in dysbaric ON [58]. Li et al also showed endothelial cell damage as well as a pro-coagulant and a low fibrinolytic milieu- as potential pathologic mechanisms of GC-induced ON [39]. Intravascular coagulation is itself an intermediary event that has both hereditary and acquired risk factors. There are two forms of plasminogen activator inhibitor (PAI). PAI-2 is a thromboblastic product and PAI-1 is a serine protease inhibitor that is synthesized and released by endothelial cells in blood vessel walls. PAI-1 exerts its regulatory activity on fibrinolysis by forming complexes with tissue plasminogen activator (t-PA). The t-PA/PAI-1 complex does not

have the ability to activate plasminogen to plasmin. Increase in PAI-1 activity suppresses the generation of plasmin resulting in hypofibrinolysis and a relative hypercoagulable state [59]. In a clinical and experimental study, the activity of t-PA and PAI-1 decreased and increased respectively in patients with ON [39]. Endothelial injury or dysfunction can activate the thrombotic cascade, followed by ischemia and infarction in the femoral head [14]. Thrombus formation can also be stimulated by other mechanisms such as the microparticles, composed of residual bodies of apoptotic endothelial cells, directly inducing endothelial dysfunction followed by activation of the thrombotic cascade. Apoptosis of endothelial cells stimulates the binding of thrombocytes to the endothelium that further induces platelet activation and thrombus formation [60].

Effects on angiogenesis and the repair mechanism

Following necrosis of the femoral head, a repair process begins with the entry of blood vessels into the necrotic region, followed by bone resorption and subsequent bone formation. Several essential factors such as vascular endothelial growth factor (VEGF), act directly on endothelial cells and induce angiogenesis [61]. Dysregulation of these essential factors will have an effect on angiogenesis, and consequently the repair process. Yang et al used VEGF gene transfection to enhance the repair of ON in a rabbit model of ON [61, 62]. It has been shown that DEXA can decrease the synthesis of VEGF protein, as measured by ELISA, by 45% in a multipotential cell line (D1) derived from bone marrow [63]. Myofibroblastic cells induce capillary formation by producing endothelial growth factor and collagen [64,65]. Harada showed that GC inhibited capillary growth significantly by suppressing collagen synthesis by myofibroblastic cells [65]. In addition, other mechanisms may impede angiogenesis. One of them appears to be related to basement membrane turnover, which is determined, in part, by proteolytic activity associated with proliferating vessels [26]. Plasminogen activators are serine proteases that convert plasminogen to plasmin and can cleave extracellular proteins, either directly or indirectly as a result of plasmin production. This alters cell-matrix interactions by liberating mitogens and angiogenic factors that stimulate endothelial migration and

proliferation [26]. Decreased fibrinolytic activity, which may be a consequence of increased PAI-1, has been described in patients with ON [10,11,66]. Inhibition of proteolytic steps involved in vessel growth may underlie, in part, the mechanism by which GCs induce ON.

In addition to GC, constituents of cartilage as well as interferons have been shown to be potential inhibitors of angiogenesis. Following subchondral fracture, the cartilage components are directly exposed to the ongoing repair process through the fracture cleft. Cartilage constituents may thus play a role in the development and/or continuance of the disease process in ON and explain the localization of ON to subchondral bone tissue [48]. ON has also been reported secondary to interferon treatment in multiple sclerosis, Crohn's disease, leukemias, and hemangiomas [54] when used alone or in association with GCs.

Action on vasoactive substances

Vascular endothelial cells regulate vascular tone through the release of relaxing and contracting factors that modulate the contractile activity of vascular smooth muscle cells. GC excess causes overproduction of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals, and thereby perturbs nitric oxide (NO) availability in the vascular endothelium. These events can lead to vascular complications in patients with GC excess. Nitric oxide (NO), an endothelial cell-derived relaxing factor, is an important vasoactive mediator for the reaction of endothelium-dependant vascular relaxation. A decrease in its availability due to perturbation of synthesis and/or release of NO by vascular endothelial cells causes an increase in vascular resistance [67].

GC excess enhances superoxide-induced inactivation of eNO (endothelial nitric oxide) and suppresses eNO production through decreasing the expression of endothelial nitric oxide synthase (eNOS). The synthesis of eNO could be influenced by GC through three potential mechanisms: direct injury effect of GC on endothelial cells, repressing eNOS activity and increasing blood lipid levels [39]. eNO has several anticoagulant actions, including dilatation of blood vessels, prevention of platelet aggregation and inhibition of monocyte adherence to the endothelium [68]. GC-induced decrease in NO

bioavailability elicits vascular endothelial dysfunction, leading to insufficiency of peripheral circulation, thus a potential mechanism for glucocorticoid-induced ON [69].

The response of isolated intraosseous femoral head arteries (lateral epiphyseal arteries which provide the major blood supply to the femoral head) to endothelin-1 was enhanced after long-term corticosteroid treatment (3 months of methylprednisolone) in an immature pig. The response to other physiological vasoactive substances such as noradrenalin, substance P nitric oxide and bradykinin was unchanged [70]. Endothelins are potent vasoconstrictors that are synthesized and released by vascular endothelial cells and that bind to vascular smooth muscle in bone. In another study by Drescher et al. [71] vasoconstriction by noradrenalin was not altered by methylprednisolone. However, bradykinin elicited a concentration-dependent vasodilatation, which was lower in the GC-treated vessels than in the non-treated vessels. In contrast, endothelin-1-induced vasoconstriction was stronger in the GC-treated vessels. Endothelin causes vasoconstriction by increasing intracellular calcium levels in vascular smooth muscle. Endothelin-1 has the strongest vasoconstrictive potential among endothelins. Hence, methylprednisolone was shown to enhance constriction of femoral head lateral epiphyseal arteries, decreasing femoral head blood flow [72].

Prostacyclin is another potent vasodilator produced by vascular smooth muscle cells and endothelial cells, and its production is decreased by GC treatment [72]. From studies on proteases involved in blood pressure homeostasis, DEXA was shown to increase angiotensin-converting enzyme aminopeptidase, while suppressing the kallikrein-kinin system. These effects resulted in enhancement of angiotensin II and angiotensin III levels which also contribute to elevation of blood pressure. The suppression of the kallikrein-kinin system (by DEXA itself and by elevation of angiotensin-converting enzyme) also has additive effect by inhibiting vasodilation [73].

Thus, GCs appear to act as regulators of local blood flow by modulating the vascular responsiveness to vasoactive substances. This phenomenon potentiates the hypertension induced by endothelial cell apoptosis as discussed above. GC-induced vasoconstriction in the intraosseous femoral head arteries reduces blood flow and causes inadequate blood supply to the femoral head.

Glucocorticoid and fat metabolism

It has been proposed that GCs produce serious intramedullary fatty infiltration [74]. The effect of this fatty tamponade in the medullary cavity is the result of lipocytes on the surface of vascular sinusoids resulting in less efficient function of vascular sinusoids and diminished vascular area in the femoral head [75]. The diminished blood flow at the femoral head level can lead to secondary necrosis [74].

In an *in-vitro* study by Li et al., DEXA-induced adipogenesis in a pluripotent marrow cell lineage (D1) cloned from BALB/c mice, was accompanied with lipid vesicle accumulation within the cells, up-regulation of the expression of adipogenic genes (AP2 and PPAR [gamma]), and down-regulation of osteogenic gene expression, type I collagen, Runx2/Cbfa1, and osteocalcin [63,76]. The effect of steroids on adipogenesis by D1-BAG, a pluripotent cell cloned from mouse bone marrow and transfected with traceable genes encoding beta-galactosidase and neomycin resistance, was investigated in *in-vitro, ex-vivo* and *in-vivo* in mice. Treatment of D1-BAG cultured cells with DEXA produced an accumulation of lipid vesicles and stimulated expression of fat cell-specific 422(aP2) mRNA. Data from the mice (*in-vivo)* study showed adipogenesis from steroid treatment in 5-9% of transplanted cells. These results indicate that steroid-induced differentiation of potentially osteogenic marrow cells into adipocytes may contribute to the development of ON [77]. It has been shown that the number of adipocytes in culture increased with longer marrow stromal cells' exposure to DEXA and the concentration of DEXA [78]. As above, data from *in-vivo* studies demonstrated that adipocytes in bone marrow increased after steroid exposure. Fat degeneration and necrosis, considered early signs of ON, were also observed [12]. Li et al. have also shown that DEXA can directly induce differentiation of marrow stromal cells into a large number of adipocytes and inhibit their osteogenic differentiation [78]. Kitajima et al. showed that mature fat cells exposed to high-dose GC were bigger than control cells both derived from bone marrow [79]. In their study, Jones et al. suggested that liquid fat, thromboplastin and other vasoactive substances released from injured marrow adipocytes in ON affect the vascular

walls (endothelial cells) and produce a hypercoagulable state through the endothelial cells [80].

Therefore, these studies suggest that GC might cause ischemic ON through elevation of intraosseous pressure, and subsequently decreased blood flow to the femoral head via adipogenesis and fat hypertrophy in the bone marrow.

Interaction with Regional Endothelial Beds

As mentioned above GC excess causes overproduction of reactive oxygen species and thereby perturbs NO availability in the vascular endothelium. The endothelial cell monolayer constitutes the inner lining of the vascular wall and plays an essential role in the homeostasis of the blood. Due to its unique localization, the endothelium is continuously exposed to inflammatory cells and circulating factors which can induce endothelial activation and/or injury [81]. The concept of a focal nature of a systemic haematological defect resulting in local hypercoagulable state is relevant to femoral head ON [54]. Clotting abnormalities often manifest in isolated endothelial beds. Deficiencies of antithrombin III, protein C and protein S result in deep venous thrombosis of the extremities. Thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome result in microthrombotic lesions that are detectable in all organs except the liver and lungs. Antiphospholipid- antibody syndrome results in clotting of only particular venous and arterial areas including the retina and the placenta. The heterogeneity of the endothelial cell structure amongst these many different organs has been postulated to be a probable cause for the varied clotting responses in the separate endothelial beds [82]. Regional endothelial beds (REBs) have a different local expression of systemic defects. This regional endothelium dysfunction may be a mechanism for ON [14].

Endothelium dysfunction or activation, primarily by endothelium itself or secondary to a stimulator, could activate the thrombosis cascade, followed by ischemia and infarction. Medications such as GCs reinforce the vascular processes leading to thrombotic occlusion. They directly injure endothelial cells and amplify hypercoagulability [37].

Risk factors and underlying disease

GC may potentiate the effects of endogenous hypercortisolism (Cushing's disease), a pre-existing condition known to increase the risk of ON [83], although ON is more likely to develop following exogenous GC administration [84]. Specific binding for [3H]DEXA with high affinity and low capacity has been demonstrated in the isolated osteoblasts, mostly binding in the nuclear fraction, with a dissociation constant (Kd) of approximately 3.3 nM [22]. The Kd of [3H]triamcinolone acetonide, another exogenous GC, was equal with 4.0 +/- 1.43 nM, reflected high-affinity binding [85]. In contrast, the Kd for GR of endogenous GC, cortisol/corticosterone has been reported to be 10-20 nM [86]. Thus, the relative binding affinities of steroid for receptor were found to be greater in exogenous than endogenous GC. The use of high doses of hydrocortisone or methylprednisolone for an extended duration was shown to be a significant risk factor for ON in patients suffering from severe acute respiratory syndrome [87]. ON has been reported with moderate to high doses of short duration steroid usage in infertility treatment [88, 89]. Amongst many other disease processes, ON has also been reported during the treatment of hay fever with corticosteroid use over a period of 10 years [90-92]. Some diseases have been reportedly associated with ON, regardless of GC treatment [54].

Amongst patients receiving a specific GC dose, only an unpredictable subset will develop ON, suggesting the presence of individual differences in steroid sensitivity and the potential presence of additional specific risk factors. A genetic risk factor for intravascular coagulation may not, by itself, provide an answer. Although the prevalence of coagulation abnormalities in patients with GC-induced ON is increased in some studies compared to controls [9,10,53-55], a considerable proportion of ON patients does not demonstrate an increased prevalence for thrombophilic disorders [56,57]. Furthermore, not all patients with both GC usage and thrombophilic factors develop ON [33]. Likewise, although alcoholism is associated with ON as an etiological factor, not all heavy alcohol users develop the disease. Therefore, it is difficult to say whether ON that occurs in patients with an underlying or predisposing factor, is actually due to the

underlying disease or risk factor rather than GC treatment or alcohol. Many of the patients with predisposing factors or underlying diseases, such as systemic lupus erythematosus or acute leukemia, also have been administered GC as part of their treatment regimen. Similarly, while most therapeutic regimens in oncology include steroids as part of the protocol or as an adjunctive measure in the control of nausea and/or prophylaxis against hypersensitivity reactions, cases of ON developing after chemotherapy (with or without steroids) or radiation therapy have been well described in the oncology literature [93-95]. As well, several investigators have reported ON developing in patients before GCs or replacement treatment regimen take place [6,96-98]. Hence, the current pathophysiological model of ON puts forward a multiple hit theory in which, with increasing number of risk factors, the risk of ON increases [99]. Amongst the many risk factors, GCs have the leading role in ON.

Conclusion

The pathogenesis of ON is multifactorial. One strategy to prevent the development of ON is to reduce the potential risk factors. For example, one such strategy is the use of an anticoagulant or a lipid-lowering agent to prevent the development of ON as shown in some animal models [68,79,100]. Experimental studies showed that combined use of an anticoagulant and a lipid-lowering agent was found helpful in preventing steroid-induced ON in rabbit models [101]. While more effort is needed to better comprehend the role of GC in ON, trial therapies involving antithrombotic drugs that interfere with the function of endothelial cells, medical interventions promoting angiogenesis, administration of lipid-lowering agents and anti-apoptotic drugs may all be useful in the treatment of patients with ON. The textbook model of ON is a multiple hit theory in which, with a greater number of risk factors, the risk of ON increases. Nevertheless, GCs being considered having a leading role in ON, newer data on GC action on endothelial cells and the regional endothelial bed dysfunction theory will hopefully shed new light on GC mechanism of action in ON. Several pathways of GC are now better understood. Future

medical and surgical management of ON may be better served by treatment modalities exploring these new important pathways.

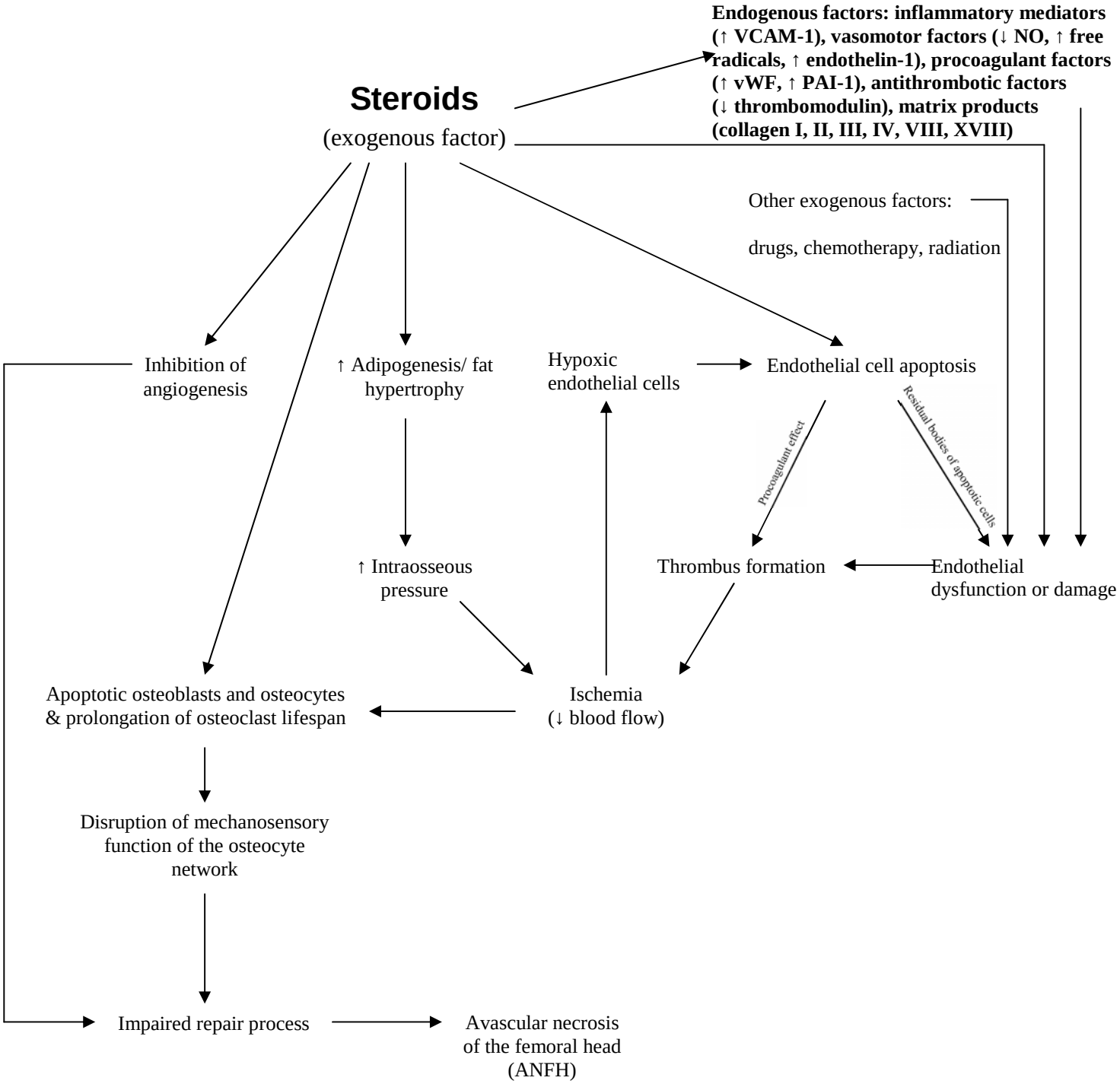

Fig. 1 Plausible mechanisms for steroid-induced ANFH development

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Chapter 3

In the next chapter, we present data on the effect of high dose dexamethasone on endothelial haemostatic gene expression and neutrophil adhesion in the presence and absence of inflammatory mediators. Glucocorticoid therapy, especially at high doses, is known to be complicated by adverse outcomes such as the development of thrombotic events or acceleration of inflammatory response in some disease conditions namely multiple myeloma and osteonecrosis. The data presented suggests that the interaction between high dose glucocorticoids, inflammatory mediators and endothelium could contribute to the development of these adverse outcomes.

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Effect of High Dose Dexamethasone on Endothelial Haemostatic Gene Expression and Neutrophil Adhesion

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Abstract

Glucocorticoid usage especially at high doses is complicated by adverse outcomes such as thrombotic events or acceleration of inflammatory response in conditions like myeloma and osteonecrosis. The mechanism(s) through which high dose dexamethasone (HDDEXA) causes vascular injury remains unclear.

We hypothesized that HDDEXA sensitizes endothelial cells (EC) to the effect of inflammatory mediators and modulates endothelial haemostatic gene expression and leukocyte adhesion. Human umbilical vein endothelial cells (HUVEC) were grown in absence or presence of HDDEXA and were also tested in presence or absence of tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS) or thrombin. mRNA and protein expression were measured and the functional consequences of HDDEXA preconditioning on cell adhesion molecules (CAM) were determined by agonist-mediated leukocyte adhesion assay.

Treatment with HDDEXA resulted in an increased induction of CAM, tissue factor and von Willebrand factor, while down-regulating thrombomodulin and urokinase. HDDEXA alone had no effect on adhesion but resulted in enhanced TNF-α- and LPS-mediated adhesion of neutrophils. Together, these findings suggest that HDDEXA sensitizes HUVEC to the effect of inflammatory mediators and induces a pro-adhesive environment in primary EC. This finding is of importance when glucocorticoid usage is required at therapeutic high doses in patients with or without thrombotic risk factors.

Keywords: dexamethasone, glucocorticoid, inflammatory, coagulation, thrombosis, endothelial cells

Introduction

Glucocorticoids (GCs) are widely prescribed in cases of rheumatoid arthritis, asthma, systemic lupus erythematosus, cancer treatment and organ transplantation. Their therapeutic advantages are offset by their well-documented adverse effects [1], especially if administered on a long-term basis. Recently, investigators have found an association between GC use and the development of venous thrombotic events [2-8] as well as an acceleration of inflammation during inflammatory disease states [2,9,10]. For instance, thromboembolic complications have been reported during the initial phase of GC use for the treatment of giant cell arteritis [7], during the treatment of minimal change nephritic syndrome [6] and also in the development of non-traumatic osteonecrosis of the femoral head [11,12].

Thromboembolic events have also been observed in patients with multiple myeloma treated with combined modality therapy including thalidomide, or its analogues [4], and dexamethasone (DEXA). In these patients, an increased risk of venous thrombosis was associated with the addition of DEXA [4,8]. In Cushing's syndrome or ACTH-dependent hypercortisolism, a hypercoagulable state has been reported [5]. In this prothrombotic condition, high levels of adrenal steroids have been shown to increase plasma or serum levels of factor VIII, von Willebrand Factor (vWF) and plasminogen activator inhibitor-1 (PAI-1) activity, with secondary decrease in fibrinolysis. Similar clotting abnormalities have been reported in patients treated with exogenous GCs [2,13].

The mechanism of the thrombogenic effect of GCs is not well understood. It has been shown that for both *in vivo* and *ex vivo* studies, lower doses of different types of GCs will initially inhibit arterial thrombosis through inhibition of platelet aggregation; whereas at higher doses, these effects are counteracted by a significant inhibition of the fibrinolytic activity [8,14-17]. This was shown to occur through decreased tissue plasminogen activator (t-PA) activity and increased PAI-1 antigen levels [14,15]. Aside from the thromboembolic effect, it is widely reported that high dose of GCs may exacerbate the inflammatory process of a disease state [2,9]. For example, it may accelerate the development of atherosclerosis, a chronic inflammatory state, as reported in systemic lupus erythematosus (SLE) [9] and also in sepsis, a process which results in activation of

inflammatory and coagulation events [10]. Moreover, it is well known that the endothelium is actively involved in the balance of the coagulation/fibrinolytic pathways. Thus, we wanted to assess the effect of high doses of DEXA on the profile of endothelial haemostatic genes involved in proinflammatory and prothrombotic events and their contribution to the adhesion of leukocytes on activated endothelial cells.

Materials and methods

Cell Culture

HUVEC were purchased from Cambrex (Walkersville, MD, USA) and cultured in endothelial basal medium (EBM-2 media (Cambrex) supplemented with microvascular additives (excluding hydrocortisone): human endothelial growth factor, 5% fetal bovine serum (FBS), vascular endothelial growth factor, human fibroblast growth factor type B, R3 insulin-like growth factor 1, ascorbic acid, heparin and the appropriate antibiotics gentamicin and amphotericin B (as per the supplier's protocol). HUVEC were also cultured in a serum-starved media (EBM-2 for endothelial cells containing 0.5% FBS, without supplement). Cells were cultured to near confluence and then incubated with or without 1 mM DEXA (Sigma Chemical, St. Louis, MO, USA) for 48 hours. The 1 mM concentration of DEXA was selected based on previous performed dose-response experiments and previous studies reporting the effects of high dose DEXA [18-22]. At the last four to twelve hours of treatment with DEXA, Tumor Necrosis Factor-α (TNF-α, 10 ng/ml; Invitrogen, Burlington, ON, Canada), Lipopolysaccharide (LPS, 10 μg/ml; Sigma Chemical), or Thrombin (Thr, 1.5 U/ml; Calbiochem, San Diego, CA, USA) was added. The concentration for the agonists were selected according to previous reports [23,24]. The maximum incubation time with TNF-α, LPS, or Thr was 12 hours, whereas for thrombomodulin, the incubation period was 4 hours as observed previously [24]. After 48 hours in culture, HUVEC were harvested following a 5 minute incubation period with trypsin (0.05%) and EDTA (0.5 mM).

Since both DEXA and serum starvation could influence the expression of genes involved in the modulation of the apoptotic pathway [25-27] HUVECs were cultured in a non-serum-starved media condition. A serum-starved media (EBM-2 for endothelial cells containing 0.5% FBS without supplement) was also tested.

Cell viability assay with trypan blue exclusion test

The cell viability of the HUVEC was assessed at 24, 44, and 48 hours following incubation with DEXA; subconfluent HUVEC were suspended in 2 ml of medium and 50 μl of this mixture was incubated with an equal volume of 0.4% trypan blue (Sigma

Chemical). Cells incorporating trypan blue were considered non-viable, and were counted using a hemocytometer, a cell counter (Coulter, Hialeah, FL, USA) and an inverted microscope (Olympus CX2; Optical Co., LTD, Tokyo, Japan). Cell viability above 95% was considered ideal and over 90% acceptable [23].

Real-Time Polymerase Chain Reaction (TaqMan RT-PCR)

HUVEC were harvested for total RNA utilizing the RNeasy Mini kit (QIAGEN Inc., Mississauga, ON, Canada) followed by DNase I treatment (QIAGEN). Multiplex RT-PCR was carried out according to the protocol provided by the manufacturer for the TaqMan One step PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA, USA). Primer-probe sets were designed with the Primer Express 1.5 software and synthesized by Applied Biosystems and a thermal cycler (Prism 7900, Applied Biosystems). All primer and probe sets were subject to database search to ensure that there was no potential conflicting transcript matches to pseudogenes or homologous domains with related genes. The sequences of primers and probes are listed in Table I. For the relative quantification of gene expression, the comparative threshold cycle (ΔCt) method was employed and normalized against 18S rRNA (primers and probes provided from Maxim Biotech, Inc. Rockville, MD, USA), which was measured by the same method. All PCR reactions were performed in triplicate. Control reactions were set up lacking reverse transcriptase to assess the level of contaminating genomic DNA.

Western blot analysis

Cells were solubilized with lysis buffer (125 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue), scraped and protein concentration determined by Bradford assay. Cell lysates were immunoprecipitated with goat polyclonal anti-human CD62E (E-selectin) IgG, rabbit polyclonal anti-human CD106 (VCAM-1) IgG, or with rabbit polyclonal anti-human CD54 (ICAM-1) IgG (Santa Cruz Biotechnology, Santa Cruz, Ca, USA) and separated on SDS-PAGE. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane and probed with the appropriate horseradish peroxidase-coupled secondary antibodies (Santa Cruz

Biotechnology). Membranes were stripped using Re-Blot Plus strong stripping solution (Chemicon International, Lake Placid, NY, USA). Immunoreactive bands were visualized using LumiGlo^{TM} (New England Biolabs, Pickering, ON, Canada). The protein expression was estimated by scanning densitometry of Western blots using a calibrated imaging densitometer and quantified using Quantity One software (Bio-Rad, Mississauga, ON, Canada).

FACS assay

Cells were incubated with FITC-conjugated antibody at 4˚C for 50 min, followed by three washes with fluorescence-activated cell sorting (FACS) washing buffer. The antibodies were the followings: mouse anti-human FITC-conjugated monoclonal CD54 (ICAM-1) antibody (ID Labs Inc., London, ON, Canada), mouse anti-human FITC-conjugated monoclonal CD106 (VCAM-1) antibody (Becton, Dickinson and Company, Mississauga, ON, Canada) and mouse anti-human FITC-conjugated monoclonal CD62E (E-selectin) antibody (ID Labs Inc.). Fluorescence data were collected based on the mean fluorescent intensity values using a Becton Dickinson FACScan (FACSCalibur). All experiments were performed at least in triplicate.

Neutrophil Isolation and Purification

Venous blood was obtained from healthy donors. The protocol was approved by the Montreal Heart Institute's ethical committee. Neutrophils were isolated as previously described [28]. Ninety-five percent of the isolated cells were polymorphonuclear cells as determined with a cell counter. Cell viability was found to be greater than 98% as assessed by the trypan blue dye exclusion assay.

Neutrophil adhesion assay

HUVEC were seeded and cultured in gelatin (0.25%)-coated 24-well plates in EBM-2 medium with the microvascular additives excluding the hydrocortisone. After 24 hours, the complete media was replaced with fresh complete media, with or without DEXA (1 mM). 24 hours later, media was replaced and a cell viability test was performed. After 20

additional hours, the three stimulators (TNF-α, LPS or thrombin) or PBS (negative control) were added in the cell media for three hours. Cells were rinsed with Hank's buffered salt solution (HBSS) plus HEPES (10 mM). Neutrophils ($1x10^5$ in 500 µl of HBSS/HEPES with 5 mM $CaCl_2$) were added to each well and incubated for 1 hour at 37°C. The wells were rinsed with HBSS/HEPES to remove nonadherent neutrophils, and fixed with a 1% paraformaldehyde-PBS. Adhesion of neutrophils to HUVEC monolayers was assessed with a color video digital camera (Sony ExwaveHAV) adapted to a binocular microscope (Olympus CX2) as previously described [29].

Statistical Analysis

Data reported on mRNA, protein expression and adhered neutrophils results were given as the mean ± SEM. Comparison between groups was made with student's t-test for parametric data and Mann-Whitney U test for non-parametric data. Differences were considered at P values less than 0.05.

Results

The mRNA expression levels of nine selected haemostatic candidate genes [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin (E-Sel), Plasminogen activator inhibitor-1 (PAI-1), tissue factor (TF), von Willebrand factor (vWF), thrombomodulin (TM), tissue-plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA)] was measured in HUVEC exposed to PBS or to high dose DEXA (1 mM) ± three different agonists (TNF-α, 10 ng/ml; LPS, 10 µg/ml or Thr, 1.5 U/ml) respectively (Figure 1A-I). Treatment with DEXA alone resulted in a significant induction of all three cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) as well as TF and vWF mRNA, while down-regulating TM and u-PA mRNA levels (P value < 0.05) but had no significant effect on basal level of PAI-1 and t-PA. In absence of DEXA preconditioning, ICAM-1, VCAM-1, E-Sel, TF and u-PA were upregulated significantly in the presence of TNF-α or LPS. Conversely, TM mRNA was down-regulated under the same experimental conditions. The t-PA gene expression was significantly downregulated only in the presence of TNF-α. Following 48 hours exposure to DEXA (1 mM) in the presence of agonists, the effects became significantly enhanced compared to DEXA alone specifically for LPS-mediated ICAM-1 and E-Selectin mRNA levels as well as TNF-α-, LPS- and Thr-mediated tissue factor or vWF mRNA levels. (Fig.1). Results under serum-starved conditions (EBM-2 for endothelial cells containing 0.5 % FBS without supplement) showed the same trend, but somewhat a lower response in general was seen except in the presence of thrombin: results showed enhanced thrombin-mediated induction of cell adhesion molecules (E-Selectin and ICAM-1) and TF under these conditions (data not shown).

Table 1. List of primers and probes used in the Real-time PCR assay.

Gene Name	Sequences (5' to 3')
h VCAM-1	Forward Primer: TCG AGA CCA CCC CAG AAT CT
	Reverse Primer: GCC TGT GGT GCT GCA AGT C
	Probe: ATA TCT TGC TCA GAT TGG TGA CTC CGT CTC A
h TBMD	Forward Primer: GCC AGA TGT TTT GCA ACC AGA
	Reverse Primer: AGC TAG CCT GGG TGT TGG G
	Probe: TGC CTG TCC AGC CGA CTG CG
h PAI-1	Forward Primer: TCGAGG TGA ACG AGAGTG GC
	Reverse Primer: CAT GCG GGC TGA GAC TAT GA
	Probe: CGG TGG CCT CCT CAT CCA CAG CT
h ICAM-1	Forward Primer: GCC AGG AGA CAC TGC AGA CA
	Reverse Primer: GGC TTC GTC AGA ATC ACG TTG
	Probe: TGA CCA TCT ACA GCT TTC CGG CGC
h t-PA	Forward Primer: AGT TCT GCA GCA CCC CTG C
	Reverse Primer: CCA CGG TAG GCT GAC CCA T
	Probe: CTC TGA GGG AAA CAG TGA CTG CTA CTT TGG G
h u-PA	Forward Primer: GCC CTA AAG CCG CTT GTC CA
	Reverse Primer: AGG AGA GGA GGG CTT TTT TCC
	Probe: TGC ATG GTG CATGAC TGC GCA
h vWF	Forward Primer: AGT GTC CCT GCG TGC ATT C
	Reverse Primer: CGG CAA ATG CAG GTG TTG
	Probe: CCC CGG CAC CTC CCT CTC TCG A
h TF	Forward Primer: TTC ACA CCT TAC CTG GAGACA AAC
	Reverse Primer: CAT TCA CTT TTG TTC CCA CCT G
	Probe : TCG GAC AGC CAA CAA TTC AGA GTT TTG A
h E-Selectin	Forward Primer: TGC CAA GCA GCA TGG AGA C
	Reverse Primer: ACC ACA TTG CAG GCT GGA AT
	Probe : CAG TGT ATG TCC TCT GGA GAA TGG AGT GCT C

h = human

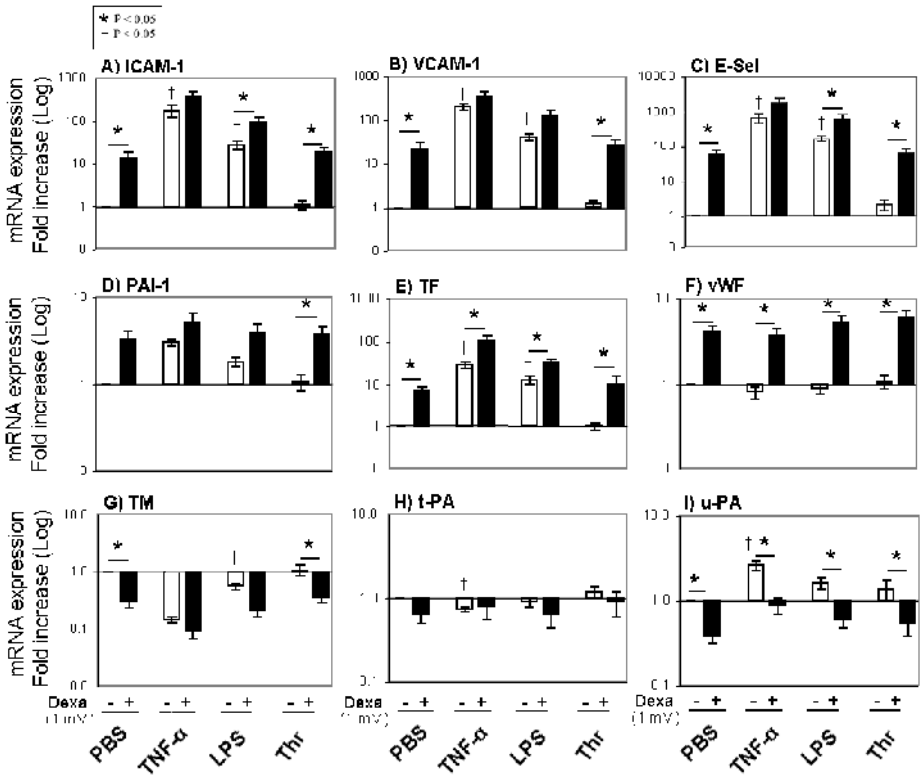

Fig 1. **Dexamethasone (DEXA) sensitizes HUVEC to TNF-α, LPS and Thr-mediated expression of haemostatic genes mRNA levels**. **A-I:** Real-Time PCR assays of **A**, ICAM-1 **B)** VCAM-1 **C)** E-Sel **D)** PAI-1 **E)** TF **F)** vWF **G)** TM **H)** t-PA **I)** u-PA mRNA expression in HUVEC preincubated for 48 hours in the absence (control) or presence of Dexa (1mM) and treated with (+) or without (-) TNF-α (10 ng/ml), LPS (10 ug/ml) or Thr (1.5 U/ml) for 4 hours. Data shown are means ± SEM of 6 experiments; * $p < 0.05$ comparing the effect of Dexa in the absence or presence of agonist and † $p < 0.05$ comparing the effect of agonist to control (PBS).

As we observed that high dose of DEXA regimen modulated haemostatic gene mRNA we wanted to assess the effect of high dose of DEXA on the expression of endothelial cell surface proteins, which have been assayed by FACS (Fig 2A-C) and Western blot analyses (Fig. 3A-C). By FACS analyses, we observed that a treatment with DEXA alone resulted in a significant induction of all three cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) (Figure 2) which are correlating with their corresponding upregulated mRNA levels (Figure 1A-C). In absence of DEXA preconditioning, ICAM-1 and E-Sel were upregulated significantly in the presence of TNF-α or LPS (ICAM-1) (Figure 2), whereas both cytokines did not promote a VCAM-1 protein upregulation (Figure 2). In addition, a treatment with Thr alone had no significant effect on protein expression of those endothelial adhesion molecules (Figure 2). Following 48 hours exposure to DEXA (1 mM) in the presence of agonists, TNF-α, LPS or Thr increased significantly the cell surface expression of ICAM-1 and E-Sel as measured by FACS ($p < 0.05$) whereas LPS and Thr increased significantly cell surface expression of VCAM-1 ($p < 0.05$) but not in the presence of TNF-α (Fig. 2A-C).

We then performed Western blot analyses selecting the same endothelial cell adhesion molecules ICAM-1, VCAM-1 and E-Selectin. Sustained treatment with DEXA (1 mM) alone enhanced ICAM-1 protein expression significantly ($P < 0.05$) but failed to promote protein expression of other cell adhesion molecules. In the absence of DEXA preconditioning, ICAM-1 and VCAM-1 protein expression were upregulated in the presence of TNF-α or LPS, whereas modulation in the presence of Thr was seen only for ICAM-1 gene. Following 48 hours exposure to DEXA (1 mM) resulted in significant increased TNF-α- mediated induction of these cell adhesion molecules whereas no significant modulation was seen in the presence of LPS or Thr (Fig. 3A-C).

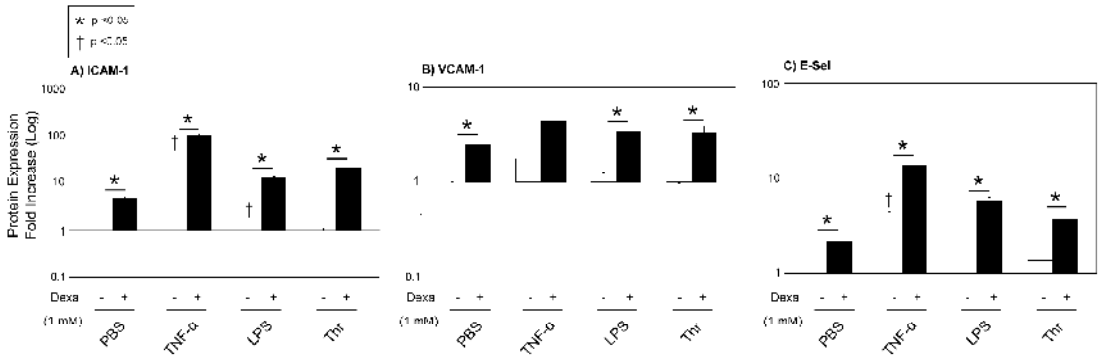

Fig 2. **Dexamethasone (DEXA) sensitizes HUVEC to TNF-α, LPS and Thr-mediated induction of ICAM-1, VCAM-1 and E-Selectin cell surface protein expression. A-C:** FACS assays of ICAM-1, VCAM-1 and E-Selectin cell surface protein expression of HUVEC preincubated for 48 hours in the absence (control) or presence of Dexa (1mM) and treated with (+) or without (-) TNF-α (10 ng/ml), LPS (10 ug/ml) or Thr (1.5 U/ml) for 4 hours. Data shown are means ± SEM of 3-4 experiments; * $p < 0.05$ comparing the effect of Dexa in the absence or presence of agonist and † $p < 0.05$ comparing the effect of agonist to control (PBS).

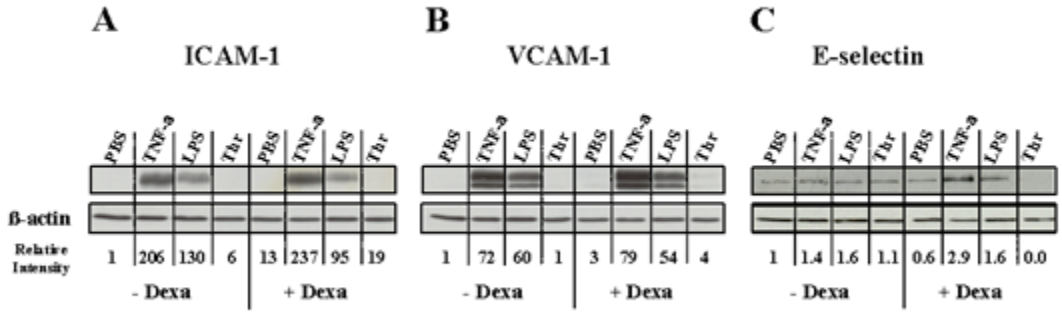

Fig 3. **Dexamethasone (DEXA) sensitizes HUVEC to TNF-α-mediated induction of ICAM-1, VCAM-1 and E-Selectin protein expression. A-C:** Western blot analysis of **A)** ICAM-1 **B)** VCAM-1 and **C)** E-Selectin protein from whole cell extract of HUVEC preincubated for 48 hours in the absence (control) or presence of Dexa (1mM) and treated with (+) or without (-) TNF-α (10 ng/ml), LPS (10 ug/ml) or Thr (1.5 U/ml) for 4 hours. Blots shown are representative of at least 3 independent experiments performed using separately prepared cell extracts. β-actin was used to monitor protein loading.

To determine the functional consequences of high dose DEXA preconditioning on cell adhesion molecules, HUVEC were assayed for agonist-mediated leukocyte adhesion (Fig. 4). Pre-treatment of HUVEC with DEXA (1 mM) for 48 hours alone had no statistically significant effect on adhesion. Treatment of HUVEC with TNF-α (10 ng/ml), LPS (10 μg/ml) and Thr (1.5 U/ml) for 4 hours resulted in a 1.98-fold, 1.97-fold, and 1.16-fold increase in neutrophil adhesion respectively without reaching significance (Fig. 4). However, following 48 hours exposure to DEXA (1mM) in the presence of agonists resulted in significantly enhanced TNF-α- and LPS-mediated adhesion of neutrophils ($p< 0.05$) (Fig. 4).

Dose-response and cell viability results

The dose-response experiment for mRNA expression of VCAM-1 using flow cytometry was performed showing the start of a plateau phase at 1 mM concentration of DEXA. Even with the dose of 1 mM in comparison to the dose of 0.1 mM the response effect did not dramatically change (nearly 20% increase)(Fig. 5). Live cell number of HUVEC under exposure to DEXA was measured. Cell viability was 95%, 93% and 92% at 24, 44 and 48 hours of exposure to DEXA and 99%, 98% and 98% in controls, respectively.

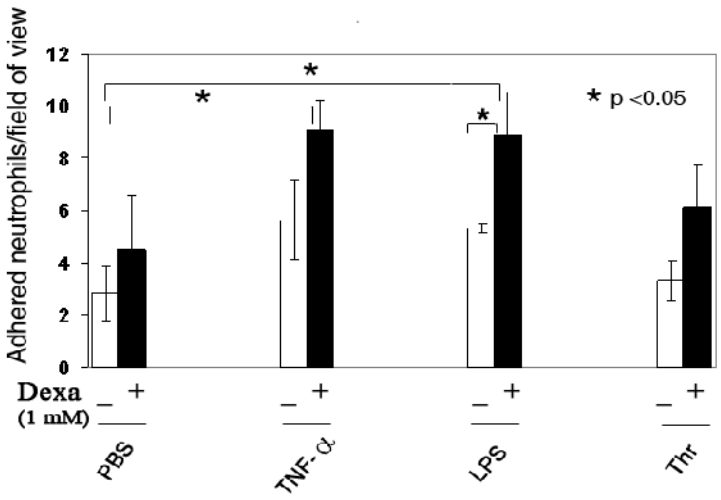

Fig 4. **Dexamethasone (DEXA) enhances significantly TNF-α and to a lesser extent LPS-mediated adhesion of neutrophils.** Confluent monolayers of HUVEC were preincubated for 48 hours in the absence (control) or presence of Dexa (1mM) and treated with (+) or without (-) TNF-α (10 ng/ml), LPS (10 ug/ml) or Thr (1.5 U/ml) for 4 hours. At the last hour of the experiment, neutrophils ($1x10^5$ in 500 µl of HBSS/HEPES with 5 mM $CaCl_2$) were added to each well, incubated for 1 hour at 37°C and adhesion of neutrophils to HUVEC monolayers was assessed. Data shown are means ± SEM of 4 experiments; ***** $p < 0.05$, comparing the effect of DEXA with (+) agonist to control (PBS) and agonist (LPS).

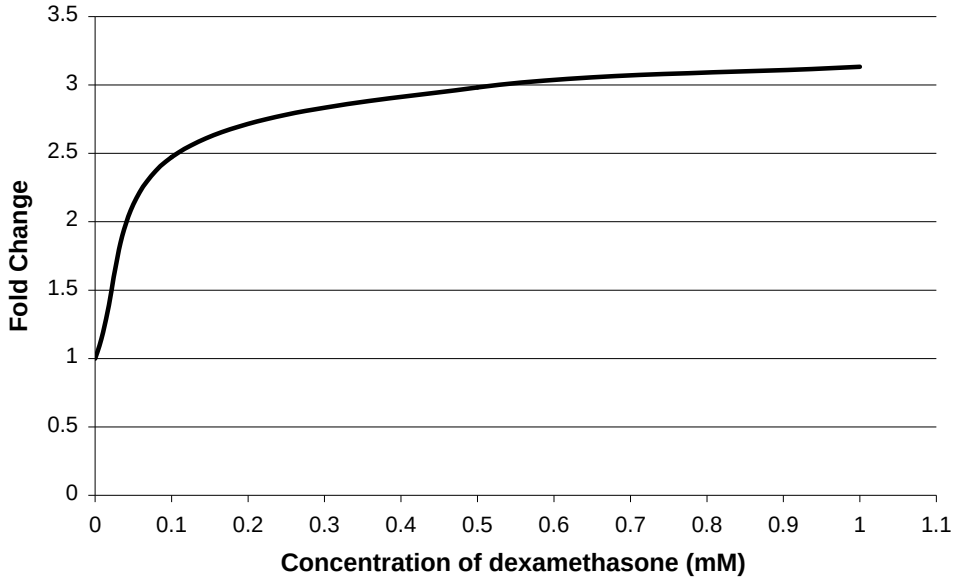

Fig 5. **Effect of dexamethasone on VCAM-1 protein expression in induced human umbilical vein endothelial cells.**

Discussion

The objective of our study was to assess the pharmacological effects of high dose of GCs on pro-adhesiveness of endothelial cells in order to better understand the mechanisms involved in the deleterious side effects of this drug at the microvascular level. GCs may cause and/or worsen outcome in diseases associated with thrombosis such as osteonecrosis of the femoral head [30], multiple myeloma [4] or stroke [31] or it may exacerbate symptoms in inflammatory processes such as sepsis [2]. These adverse effects have been reported mostly with high dose administration of GCs.

A recent meta-analysis comparing low- and high- dose of GCs treatment in patients with sepsis demonstrated a decreased survival rate with a short course of high-dose treatment, but increased survival rate and shock reversal at lower doses [2,15]. It has been thought that during sepsis the high levels of cytokines could interact with the upregulated cytokine receptors caused by GCs [2,32]. Jilma et al. also showed that high-dose (1.0 mg/kg/bid) compared to low-dose (0.04 mg/kg/bid) administration of DEXA to healthy individuals enhanced the levels of serum vWF and soluble P-selectin resulting in the development of a hypercoagulable state [13]. Therefore, it is assumed that the treatment dose of DEXA may play a major role in the outcome of the disease.

Since the scope of this study is the adverse effects caused by the high dose of DEXA, we selected a dose of DEXA of 1 mM in our *in vitro* studies, mimicking the high dose used under clinical conditions [33-37]. The concentration of DEXA in patient serum with a regular therapeutic setting (0.5 to 9 mg a day) is equivalent with 0.01-0.1 mM [22]. A dose-response experiment was performed showing the start of a plateau phase at 1 mM concentration. The concentration of DEXA (1 mM) did not produce significant toxicity as assessed by cell number and viability (based on morphology and trypan blue exclusion test).

In this study, high dose DEXA stimulated the expression of genes encoding the adhesion molecules (ICAM-1, VCAM-1 and E-selectin) mediated by TNF-α, LPS or Thr. At low dose of DEXA (1 µM) added 1 h before HUVEC activation with TNF-α or IL-1ß, only mRNA expression level of E-selectin was significantly upregulated ($p<0.05$) amongst the adhesion molecules (ICAM-1, VCAM-1 and E-selectin) [38]. In our study,

consistent with the upregulation of mRNA levels, protein expression and cell surface expression of adhesion molecules were maximal under TNF-α exposure. Several studies have shown that cell adhesion molecules, expressed by activated endothelial cells, are responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of inflammatory cells to the endothelium [39]. Incubation of endothelial cells with other cytokines such as IL-8 did not significantly alter the expression of the adhesion molecules (ICAM-1, VCAM-1 and E-selectin) [40] although, it has been shown that DEXA could effect IL-8 and inhibit its production in different endothelial cell lines [41]. Similarly, DEXA had a small inhibitory effect on constitutive monocyte chemotactic protein-1 (MCP-1) mRNA expression, but no effect on the induction by TNF-alpha [42].

Our results showed that TNF-α, LPS or Thr treatment alone increased neutrophil adherence to endothelial surface, without reaching statistical significance. Pre-treatment of HUVEC with DEXA resulted in enhanced agonist-mediated induction of neutrophil adhesion, and reached statistical significance with TNF-α and LPS ($p<0.05$). The functional assay results suggest that high dose DEXA increased the expression of adhesion molecules by endothelial cells in the presence of inflammatory mediators. Hence, high dose DEXA, in addition to enhancing the cytokine receptors, appears to exacerbate the local endothelial inflammatory process. Some studies using lower doses of DEXA showed that GCs inhibit the expression of endothelial cell adhesion molecules [25,39]. These findings may well be explained by a dose-dependent mechanism, which varies according to studies and different cell types. It has been shown that endothelial cells in different anatomic regions of the same patient are structurally and functionally dissimilar [43-46]. This regional endothelial cell dysfunction may be the disease pathway for several different disease processes where systemic genetic profiles interact with regional endothelial gene function and protein expression. One such disease process is osteonecrosis, or avascular necrosis, of the femoral head. The endothelial cell as the final common pathway for this disease has been postulated in the literature. The authors assume that the endothelial cells located in the feeding vessels of the femoral head act and may respond differently to stimuli than do endothelial cells elsewhere in the same patient [3] resulting in local microthrombi and hypofibrinolysis. This possibility is worthy of

further consideration in other disease processes, as thromboembolism of the microvasculature may be the end result where high dose GCs are known to be a significant risk factor in the disease [47].

It is also well established that inflammatory pathways closely interact with fibrinolytic and coagulation pathways. In a human endotoxemia model, GC administration to healthy subjects reduced levels of proinflammatory cytokines (TNF-α) while at the same time moderately enhancing the procoagulant response [48]. In the present study, the expression of TM was downregulated. Concurrently, the mRNA expression of TF, and vWF increased significantly under DEXA exposure. Previously, it has been shown that DEXA enhances TF expression in human monocytes [49] although results were controversial in another study [50]. High dose DEXA also increased the expression of PAI-1 under the influence of Thr. PAI-1 is an inhibitor of tissue plasminogen activator, which induces resolution of blood clots during wound healing and following inflammatory reactions [51]. Zonneveld and his colleagues showed that DEXA could increase the promotor activity of PAI-1 [52]. In our study, PAI-1 mRNA levels were upregulated significantly under DEXA exposure when cells were stimulated with Thr but not with TNF-α or LPS, although Yamamoto et al. showed that lower dose of DEXA (10^{-6} *M*) increased significantly the expression of PAI-1 gene in HUVECs stimulated by TNF-α [53].The expression of t-PA, the primary initiator of fibrinolysis, was downregulated with DEXA treatment although this did not reach significance. The expression of u-PA, another profibrinolytic factor, was also decreased when HUVEC were exposed to DEXA alone or treated with DEXA and the agonists, although interestingly, the agonists alone separately had an upregulatory effect. Based on these findings, it may be that high dose DEXA induces a hypofibrinolytic state on vascular endothelial cells.

The mechanisms underlying high dose GC-mediated sensitization of the endothelium remain to be defined. Some published data suggests a role for NF kappa-β and p38MAPK [54-56], emphasizing the importance of low-dose versus high-dose DEXA in promoting an anti-inflammatory condition since low-dose has been shown to inhibit NF kappa-β. Previous reports indicated that low dose DEX A (0.1- 1 μM) inhibits NF kappa-β binding to DNA in some cell lines such as monocytes and lymphocytes [57],

but not in endothelial cells [58] by increasing transcription of the I kappa-β alpha gene thereby increasing the concentration of this inhibitor in the cytoplasm and preventing the translocation of NF kappa-β to the nucleus [57]. Thus, the effects of DEXA especially in high dose on endothelium could possibly be explained by pathways besides the NF-kβ-dependent pathway.

In summary, these findings suggest that GCs, especially at high dose, sensitize human primary endothelial cells to TNF-α- and LPS- mediated neutrophil adhesion and modulate haemostatic gene expression favoring proadhesiveness. These results are in accord with the current conception of what the effects of GCs are at the disease level. This may provide some insight on the association between high dose DEXA and thrombotic/inflammatory events seen in clinical practice. Identification of the mechanism(s) by which high dose DEXA stimulates the expression of adhesion molecules and modulate haemostatic gene expression will be an important focus for future research, with major implications in the development of preventive strategies aimed at avoiding these complications.

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Amendments:

Real-Time Polymerase Chain Reaction (TaqMan RT-PCR)

Primer-probe sets were designed with the Primer Express 1.5 software and synthesized by Applied Biosystems and a thermal cycler (Prism 7900, Applied Biosystems, Foster City, CA, USA) was used.

Statistical Analysis

Data reported on mRNA, protein expression and adhered neutrophils results were given as the mean ± SEM. Comparison between groups was made with Student's t-test. For the small size samples Mann-Whitney U test was used since normal distribution of data was not assumed. Differences were considered significant at P values less than 0.05.

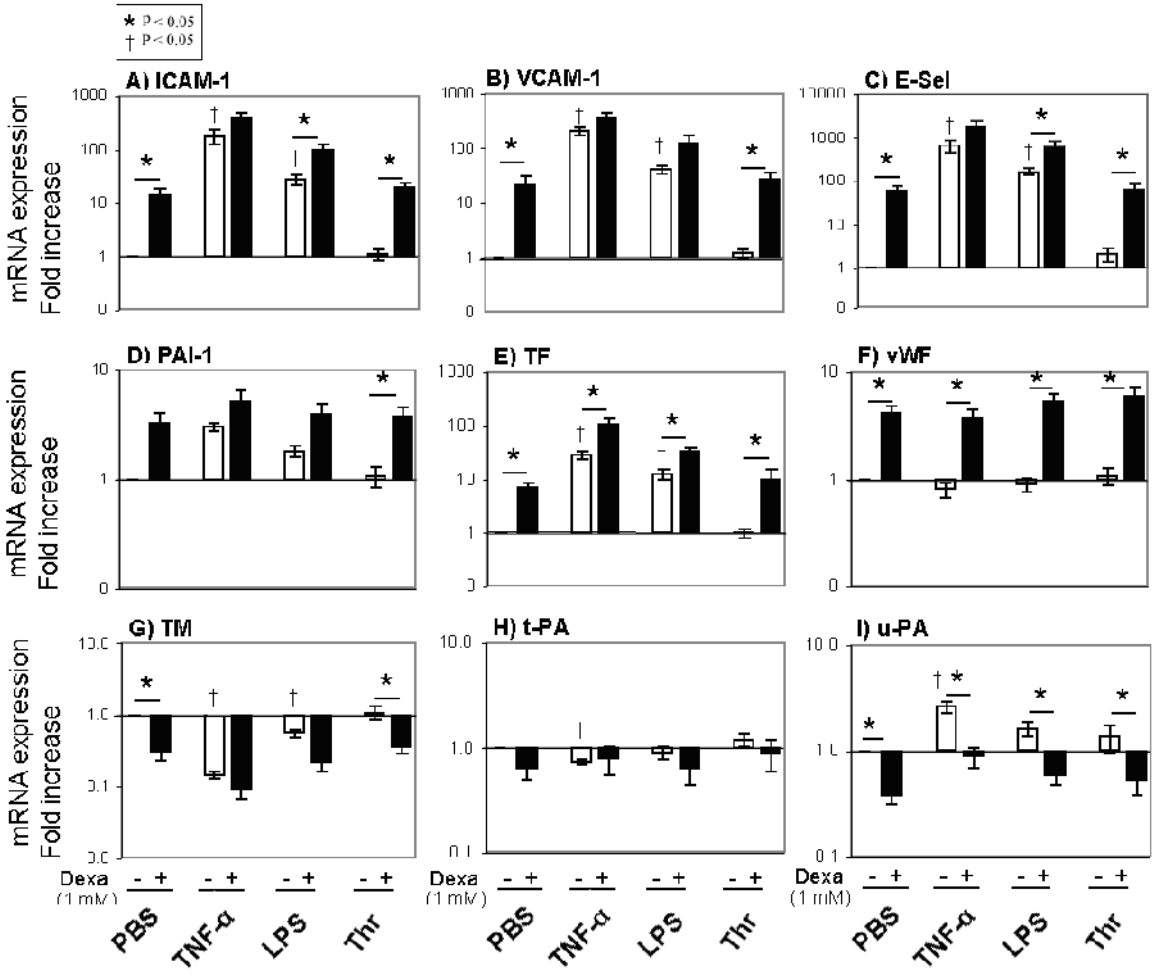

Fig 1. **Dexamethasone (DEXA) sensitizes HUVEC to TNF-α, LPS and Thr-mediated expression of haemostatic gene transcript levels**. **A-I:** Real-Time PCR assays of **A**, ICAM-1 **B)** VCAM-1 **C)** E-Sel **D)** PAI-1 **E)** TF **F)** vWF **G)** TM **H)** t-PA **I)** u-PA mRNA

expression in HUVEC preincubated for 48 hours in the absence (control) or presence of Dexa (1mM) and treated with (+) or without (-) TNF-α (10 ng/ml), LPS (10 ug/ml) or Thr (1.5 U/ml) for 4 hours. Data shown are means ± SEM of 6 experiments; * $p < 0.05$ comparing the effect of Dexa in the absence or presence of agonist and † $p < 0.05$ comparing the effect of agonist to control (PBS).

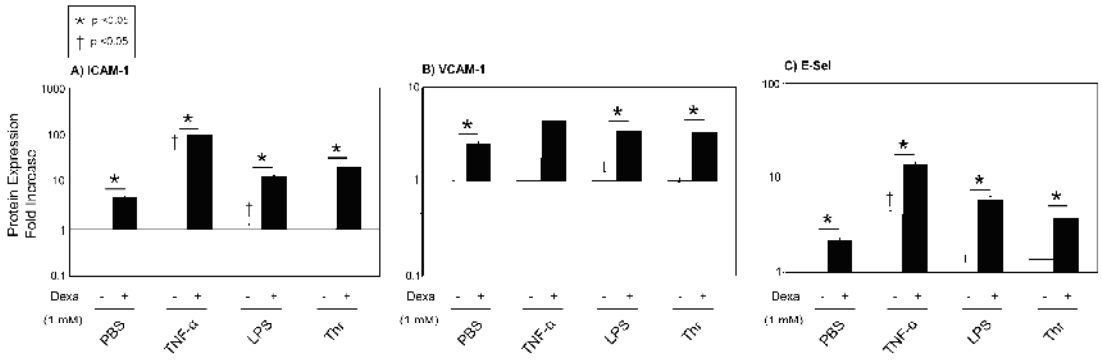

Fig 2. **Dexamethasone (DEXA) sensitizes HUVEC to TNF-α, LPS and Thr-mediated induction of ICAM-1, VCAM-1 and E-Selectin cell surface protein expression. A-C:** FACS assays of ICAM-1, VCAM-1 and E-Selectin cell surface protein expression of HUVEC preincubated for 48 hours in the absence (control) or presence of Dexa (1mM) and treated with (+) or without (-) TNF-α (10 ng/ml), LPS (10 ug/ml) or Thr (1.5 U/ml) for 4 hours. Data shown are means ± SEM of 3-4 experiments; * $p < 0.05$ comparing the effect of Dexa in the absence or presence of agonist and † $p < 0.05$ comparing the effect of agonist to control (PBS).

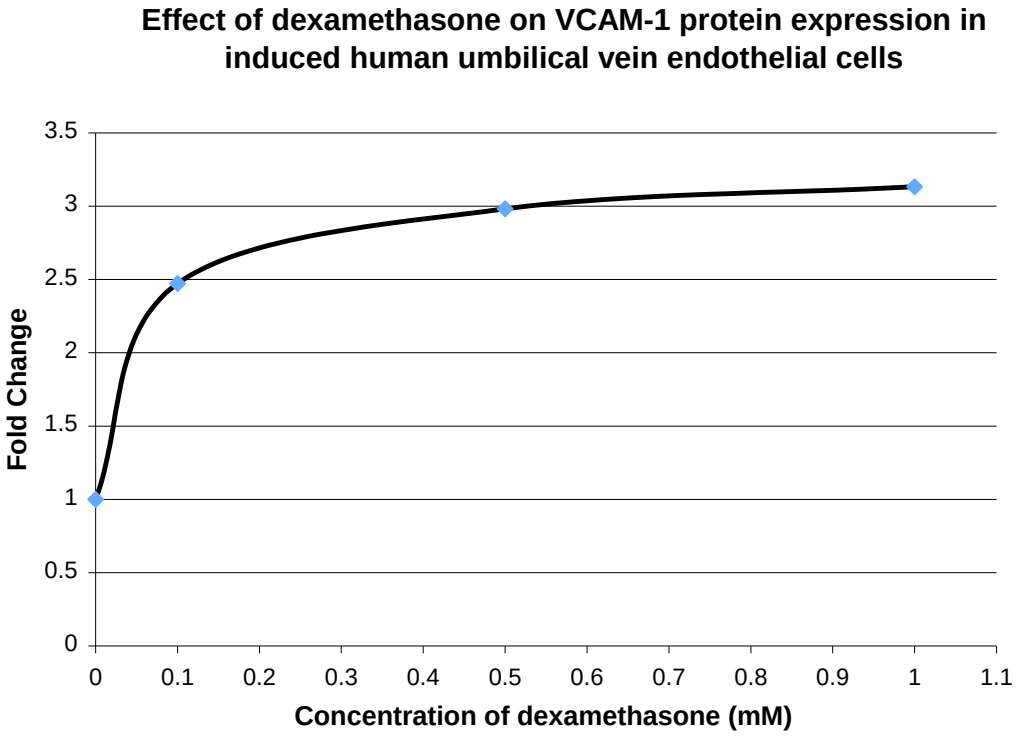

Fig 5. **Effect of dexamethasone on VCAM-1 protein expression in induced human umbilical vein endothelial cells.**

Chapter 4

Early events of ANFH are incompletely understood. ANFH is often diagnosed late, occasionally by routine follow-up investigations. Besides, bone tissue is not readily accessible to sampling. As a result, pathogenic studies rely chiefly on epidemiological data and animal models. Although epidemiological studies may detect factors associated with ANFH, they cannot prove causality. On the other hand, it is difficult to induce ANFH in animals especially in mice and rats. Aggressive methods must be used, such as total devascularization or massive glucocorticoid doses. Even then, the potential for healing is far greater in animals than in humans. In 1987 Gosling et al. suggested that neither rabbit nor rat is useful as an animal model of steroid induced osteonecrosis and suggested that further research might more profitably be directed at other species (15). Later, Chen et al. established osteonecrosis in rat with the administration of glucocorticoids but they had to excise two legs and the tail (9). Only recently, Yang et al. have developed a mouse model for glucocorticoid-induced osteonecrosis (43).

We performed a pilot study with the aim of developing a glucocorticoid-induced rat model of osteonecrosis. The goal of this study was to establish a rat model to study gene profiles of femoral head cells especially endothelial cells located at the femoral head.

Steroid-induced ANFH has been described in mature Japanese white rabbits (Kbs-JW) (27) but the genome of rabbit has been incompletely sequenced, thus limiting the usefulness of that model for the identification of genes affecting the risk of developing ANFH. ANFH has also been described in spontaneously hypertensive rats (31), but the relevance of this model to ANFH occurring in humans is unclear as there is no known association between hypertension and ANFH in humans.

Status: prepared for submission

A Rat Model for Glucocorticoid-induced Osteonecrosis

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Abstract

We evaluated groups of six rats from each of five inbred strains (Spontaneous Hypertensive Rat, Wistar Kyoto, Wistar Furth, SASCO Fisher and Lewis). Prednisone pellets (dosage of 1.82-2.56 mg/kg/day, mean: 2.26 mg/kg/day, SD: 0.19) were implanted subcutaneously for 90 days in 5 out of 6 rats in each group. After 90 days, the femurs were resected and examined histologically, and some radiographically as well. The histological examination was performed using H & E staining and included apoptosis assay (TUNEL assay). H & E staining was used to reveal the femoral head lesions, such as osteonecrosis, abnormalities of articular cartilage (cartilage nodules), and growth plate (disruption, thinning and discontinuation of growth plate). The most remarkable changes in H & E staining were seen in the Wistar Kyoto group. In this group 80% (4 out of 5) of steroid-induced rats revealed growth plate disruption with skip areas of cells. TUNEL assay revealed that 80% of Wistar Kyoto rats (4 out of 5), 100% of Wistar Furth (5 out of 5), 50% of Lewis (2 out of 4) and 100% spontaneous hypertensive rat (2 out of 2) had apoptotic osteocytes in trabeculae, whereas none of the Fisher rats (0%) showed apoptotic osteocytes. We postulated that Wistar Kyoto, Wistar Furth and spontaneous hypertensive rats were the most susceptible strains of rats to develop osteonecrosis of the femoral head while Fisher rats were resistant.

Key word: osteonecrosis, apoptosis, growth plate, rat, glucocorticoid, steroid

Introduction

Glucocorticoids (GC) are widely prescribed in cases of rheumatoid arthritis, asthma, systemic lupus erythematosus, cancer, organ transplantation and many other medical conditions. The therapeutic use of glucocorticoid has been strongly influenced by marked side effects, especially with long-term usage of this drug. The adverse effect of GC on bone has been recognized for more than 60 years [1]. The bone disease effect is characterized by decreased bone formation and *in situ* death of isolated segments of bone which are associated with osteonecrosis (ON). ON predominantly affects the femoral head and gradually progresses to fracture of the subchondral bone, collapse of the femoral head surface, and hip destruction. Although ON has been linked to a variety of conditions, including trauma, increased consumption of alcohol and sickle cell anemia, steroid administration remains the predisposing factor most commonly associated with the development of ON. There is considerable interest in identifying which patients are at highest risk for ON, with the long-term goal of modifying regimens to decrease the risk of adverse effects of therapy. Despite the strong association of steroid administration with ON, the role of potential underlying risk factors such as hyperlipidemia, thrombophilia, and hyperfibrinolysis in the circulatory system remains unclear. It has been clear that among patients receiving a specific GC dose, only an unpredictable subset will develop ON, which underscores the existence of individual variability in the action of GC and the potential presence of additional mechanisms and/or risk factors such as a genetic predisposition. On the other hand, studying the clinical pathology of ON in the early disease stage is extremely difficult in patients. Thus, animal experiments are needed to elucidate the pathophysiology of the disorder. Having a suitable animal model would allow for the systemic evaluation of host-related, including genetic variations, as well as acquired, such as treatment-related, risk factors. Glucocorticoid- induced ON has been induced in bipedal animals (e.g., chickens, emus) [2;3] and recently, in BALB/cJ mice [4]. To date, there has been no rat model of glucocorticoid-induced osteonecrosis unless it has been combined with a surgical procedure [5], which does not really model the much more prevalent non-traumatic osteonecrosis. In this study, our goal was to establish a rat model of glucocorticoid-induced osteonecrosis by screening different strains of rats in

order to uncover those whose constitutive phenotype might predispose to the development of ON.

Materials and methods

Maintenance and experimental animals

In this pilot study, retired breeder (aged 6-8 months) Fisher, Lewis, Spontaneous Hypertensive Wistar Kyoto, and Wistar Furth rats (6 of each strain) were obtained from Charles River Laboratories (Pointe-Claire, QC, Canada). All rats were kept in plastic cages (2 animals per cage) under standard laboratory conditions with a 12-hour dark/12-hour light cycle, a constant temperature of 20°C, and humidity of 48%. All rats were fed on a standard rodent diet. The rats were weighed at the beginning of the experiment and daily during the first 4 days after glucocorticoid pellet implantation, then weekly until the end of the experiment. The McGill animal care department approved the animal protocol.

Glucocorticoid administration

Slow-release prednisone pellets (Innovative Research of America, Sarasota, Florid, USA) were implanted subcutaneously with a dose release ranging from 1.82-2.56 mg/kg/day (mean: 2.26, SD: 0.19) for a 90-day period in 5 inbred rats composing each group (Fisher, Lewis, Spontaneous Hypertensive, Wistar Kyoto and Wistar Furth). Thus, each group had 5 steroid-induced rats along with 1 control rat in each group not treated with prednisone (control rat did not receive a placebo pellet).

Histological Examination

The rats were euthanatized with an overdose of ketamin/xylazine following treatment with steroid or placebo. Tissue samples were obtained from the distal femur only. Bone samples were fixed in 10% neutral buffered formalin overnight, then decalcified in 4% ethylenediamine tetraacetic acid (pH 7.2) (Sigma-Aldrich, St. Louis, MO, USA). The specimens were processed routinely and embedded in paraffin. Tissue sections were cut parasagitally with a rotary microtome to obtain 4 to 5 microns thickness, stained with hematoxylin and eosin (H & E) and evaluated by light microscopy.

Tissue samples were analyzed in a blinded fashion by an experienced bone pathologist (A.N.). Steroid-induced osteonecrosis was diagnosed based on bone and growth plate changes. The histological findings of an established ON were defined as dead trabeculae exhibiting empty lacunae with or without appositional bone formation [6]. The growth plate changes were considered as thinning, discontinuity pattern and disruption of articular cartilage alignment or growth plate alignment.

Tissue sections were also examined according to the criteria of Arlet *et al*. namely presence of degeneration, necrosis, and disappearance of marrow cells as well as the nuclear disappearance and hypochromasia of trabecular osteocytes as early signs of ON [7].

Measurement of apoptosis in undecalcified bone section

We used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay) to detect DNA fragmentation by labeling the terminal end of nucleic acids. *In Situ* Cell Death Detection Kit was obtained from Roche (Germany). TUNEL assay on paraffin-embedded tissue sections was performed as recommended by the manufacturer. Briefly, after deparaffinization and permeabilization of the tissue sections with proteinase K, the slides were incubated with the TUNEL reaction mixture containing TUNEL-Enzyme solution and TUNEL-Label solution for 1 hour at 37 °C inside a humidified chamber. After washing steps, samples were analyzed under a fluorescence microscope (in a drop of 1X PBS).The excitation wavelength ranged between 450-500 nm whereas the detection wavelength ranged between 515-565 nm (green).

DNase I-treated tissue section was used as a positive control. Negative controls for the study constituted of sample slides processed using the same procedure but only treated with TUNEL-Label solution.

Faxitron X-ray

Faxitron x-ray analysis was performed on the group of Wistar Kyoto rats (5 rats, 10 femur head samples) (Model MX-20).

Statistical Analysis

Comparison between groups was made with Fisher's Exact test. Differences were considered at P values less than 0.05.

Results

Growth plate changes were observed only in Wistar Kyoto and Wistar Furth rats (Fig. 1). Osteocyte necrosis and empty lacunae were not detected in any samples. The most remarkable changes in H & E staining were seen in Wistar Kyoto group. In this group 80% (4 out of 5) of steroid-induced rats revealed growth plate disruption with skip areas of cells. TUNEL assay revealed that 80% of Wistar Kyoto (4 out of 5), 100% of Wistar Furth (5 out of 5), 50% of Lewis (2 out of 4) and 100% Spontaneous Hypertensive rats (2 out of 2) had apoptotic osteocytes in trabeculae, whereas none of the Fisher rats (0%) showed apoptotic osteocytes (Table 1, Fig.2). In the Lewis group, apoptosis of osteocytes and osteoblasts without any degeneration of the growth plate was observed. Bone marrow and chondrocyte apoptotic cells were seen in all strains of rats. There were no signs of inflammation and necrosis, such as hyperemia, round cell infiltration, or lipid cyst formation in the slides. Plain x-rays obtained from Faxitron analysis did not revealed any significant anomaly. Thus, plain x-rays are not a suitable method to diagnose early stages of ANFH in rats.

There was an overall mortality rate of 30% among the rats in our pilot study related to the development of steroid-induced hyperglycemia in these "older" rats (a two to three times fold increase compare to control rats).

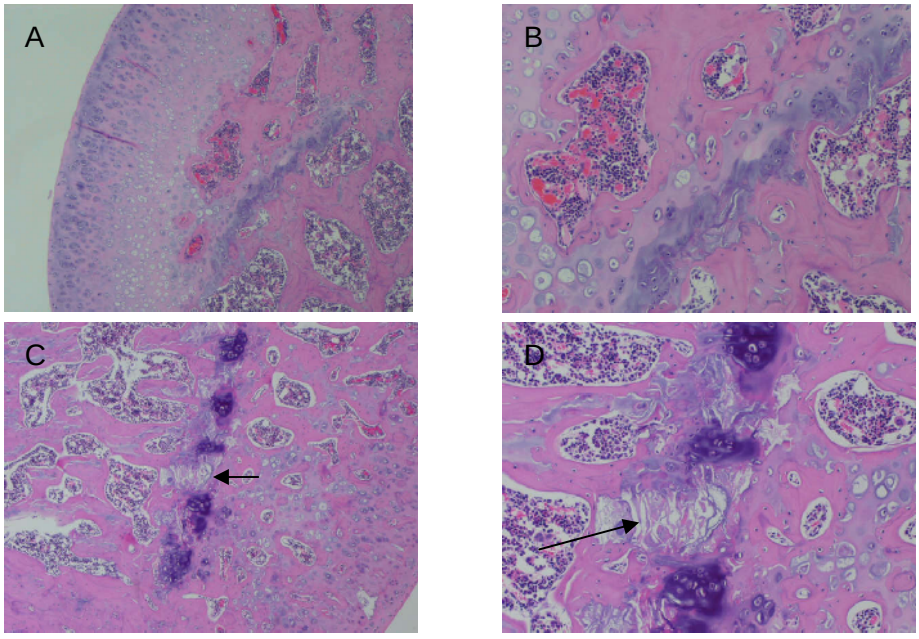

Figure 1. H & E staining in a placebo or control rat (A, B) and a steroid-induced rat (C, D). Discontinuity pattern of the growth plate is shown (arrows). Magnification A, C x100 & B, D x200.

Table 1. Apoptosis at the level of the femoral head of steroid-induced inbred rats from 5 different strains (BM: bone marrow, SHR: spontaneous hypertensive rat, WKY: wistar kyoto, WF: wistar furth)

Strain	**BM**	**Osteocyte**	**Chondrocyte**
Lewis	4/4	2/4*	4/4
SHR	2/2	2/2*	2/2
Fischer	5/5	0/5*	5/5
WKY	5/5	4/5*	5/5
WF	5/5	5/5*	5/5

*Fisher's Exact Test was significant (P value =0.0039).

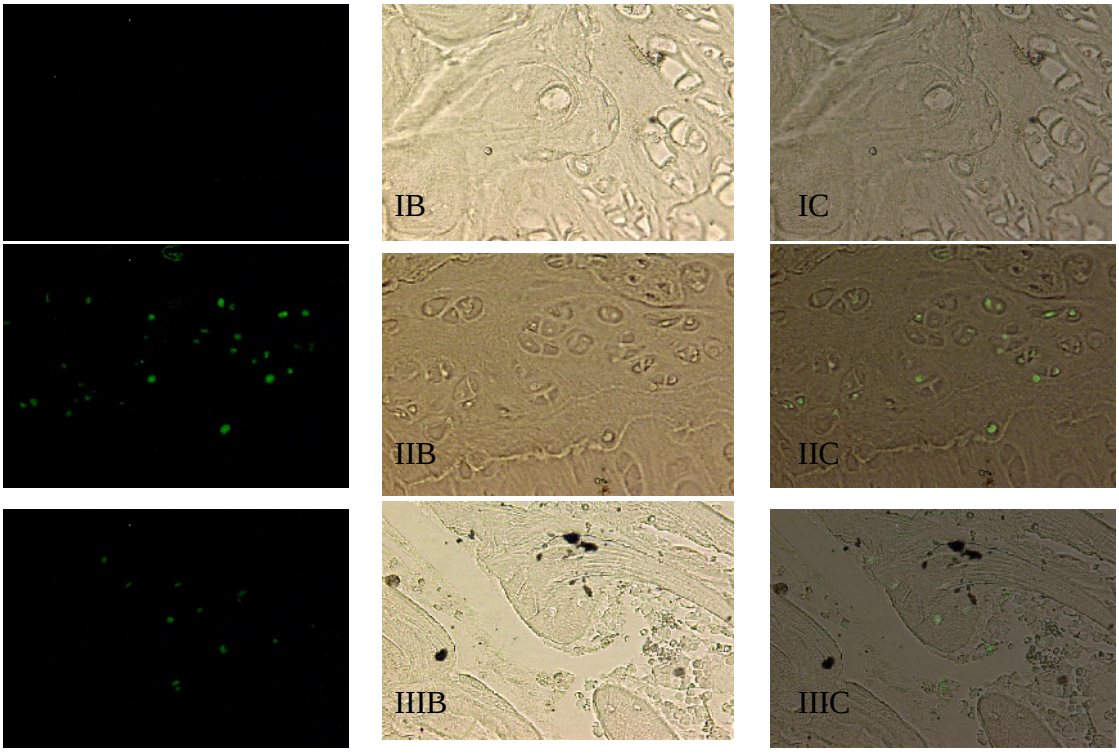

Figure 2. TUNEL assay performed on the femoral head of a female Wistar Kyoto rat treated with steroids for 3 months (II & III) compared with control Wistar Kyoto rat (I). The nucleus of apoptotic cells are shown in green. X200.

Discussion

Trueta and Amato used animal models and showed that the blood supply to the cartilage of the growth plate of the femoral head originates from the epiphyseal vessels [8], while the metaphysis is supplied by metaphyseal vessels and nutrient arteries coming from the medullary cavity. Mechanical damage to the metaphyseal arteries lead to destruction of the growth plate and, eventually, a physeal bridge [9]. It is possible that thrombosis in the methaphyseal arteries reported in osteonecrosis of the femoral head could cause injury and disruption of the growth plate with areas lacking normal cells. In our pilot study, growth plate disruption has been seen particularly in Wistar Kyoto rats. It seems that evidence of growth plate degeneration occurs in the early stage of the disease. Sato et al. have also shown that apoptosis tended to occur in early stages of ON [10]. In their rat study of ischemic ON, apoptosis occurred 12 hours after the mechanical insult, whereas no evidence of apoptosis remained after 96 hours, at which time only empty lacunae were detected [10]. They postulated that the mechanism of cell death involved in ischemic osteonecrosis was apoptosis as indicated by DNA fragmentation and the presence of apoptosis bodies in osteocytes [11;12]. Further studies have shown that apoptosis of osteocytes and osteoblasts is an important process in developing ON, especially in the early stages of ON [13]. Likewise, Kabata and his colleagues demonstrated extensive apoptosis in a rabbit model of steroid-induced ON [14]. Shibahara et al. also reported the presence of a large number of apoptotic osteocytes around necrotic areas [15].

In our study, we observed that apoptosis occurred at the level of osteocytes, osteoblasts, bone marrow cells and chondrocytes in the early stages of steroid-induced osteonecrosis-like lesions (ONLL) in three strains of inbred rats: Wistar Kyoto, Wistar Furth and Spontaneously Hypertensive. Apoptosis could result from a direct effect of steroids on the cells or could be secondary to the dysfunction/stimulation of other cells such as endothelial cells on the bone cells. Fisher rats were resistant to the development of any ONLL in response to steroid induction.

Hence, based on our findings, Wistar Kyoto, Wistar Furth and Spontaneously Hypertensive rats are the most susceptible and Fisher rats are resistant to develop

osteonecrosis of the femoral head. There were no signs of inflammation and necrosis in the samples representing the early stage of the disease. It is possible that extended exposure to glucocorticoids could establish the later stages of the disease.

Given the oberved inter-strain variability of susceptibility to the development of steroid-induced ONLL, we postulate that genetic factors are involved in osteonecrosis developing in response to steroids. Identifying these genetic factors may prove relevant to the human disorder and facilitate the identification of individuals at increased risk of developing osteonecrosis.

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Chapter 5

We postulate that hypercoagulability is playing a role in the development of ANFH at the level of the microvascular environment, implying that a thrombotic process is responsible for the interruption of blood supply to the femoral head. Even though a thrombotic process underlying the development of this disease seems appealing, we know from a clinical point of view that ANFH patients usually do not develop macrothrombosis in other vascular-bed specific regions of the body and are only very rarely affected by any of acquired of inherited classic thrombophilia. Therefore, the thrombotic process in ANFH, does not appear to be explained by the traditional thrombophilic abnormalities encountered in most thrombotic disorders. It is postulated that defects at the level of the fibrinolytic system and endothelium might be responsible if no traditional thrombophilic cofactors are found. Given the documented heterogeneity of endothelial cells originating from different vascular beds, studying endothelial cells specifically sampled from the femoral head might prove important in understanding their contribution to the development of ANFH. The following publication constitutes the first report of isolation and expansion of ECs from human bone tissue. Isolation of hBDECs in human vascular bone diseases may facilitate the study of the molecular and/or genetic abnormalities in the vasculature system that contributes to the initiation and/or progression of the disease.

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Isolation and Characterization of Human Bone-Derived Endothelial Cells

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Abstract

Historically, the etiology of local bone pathologies, such as avascular necrosis, has been related to intravascular occlusion. Recent reports have highlighted the occlusion of arteries, venules and/or capillaries in bone tissue. Endothelium of bone presumably participates locally in the formation of the microvascular thrombosis. It is also known that endothelial cells (ECs) play a central role in angiogenesis, a process seen in osteosarcoma, amongst other bone diseases. Given the well recognized heterogeneity of ECs throughout the body, investigations of local bone disease related to endothelium processes may be more appropriately targeted on bone ECs rather than other primary ECs or an immortalized EC line. In the current study, mechanical and enzymatic methods are described to isolate ECs from cancellous human bone tissue followed by immunomagnetic bead separation to purify the cell populations. The human bone-derived endothelial cells (hBDECs) were characterized based on endothelial cell antigen expression and functional assays. This study is the first report of isolation and expansion of ECs from human bone tissue. Isolation of hBDECs in human vascular bone diseases may facilitate the study of the molecular and/or genetic abnormalities in the vasculature system that contributes to the initiation and/or progression of the disease.

Keywords: endothelial, immunomagnetic bead, vascular bone disease

Introduction

Endothelium is a continuous single cell layer of endothelial cells (ECs) forming the innermost layer of blood vessel walls. These blood vessels are evident in all tissue including bone. The health of the endothelium impacts on many important physiological and pathological processes in bone. These include angiogenesis, inflammation, blood coagulation, tissue repair, and tumor growth (Gibbons and Dzau 1994; Mantovani, Bussolino, and Introna 1997). Osteonecrosis, bone metastases, primary bone tumors, healing and bone growth are some of the skeletal events that are potentially affected by EC responses. There has been a longstanding interest in the role of ECs in the development of vascular diseases (Favre et al. 2003; Franscini et al. 2004; Madden et al. 2004; Patel et al. 2003).

During embryonic development, ECs develop organ specific properties (Garlanda and Dejana 1997; Schmeisser and Strasser 2002). This results from the unique environment of each organ, as determined by exposure to physical and soluble factors, extracellular matrix, and cell interactions (Garlanda and Dejana 1997; Augustin, Kozian, and Johnson 1994). Therefore, ECs in different anatomic regions are structurally and functionally heterogeneous (Aird 2003; Augustin, Kozian, and Johnson 1994; Garlanda and Dejana 1997; Paunescu et al. 2003). An extensive number of concepts in vascular biology have relied on the *in vitro* behavior of human umbilical vein endothelial cells (HUVECs) (Bagley et al. 2003). Aside from the organ-specific differences among ECs, HUVECs are derived from a macro-vascular bed that does not exist in adults. In many vascular diseases involving inflammation and/or microvascular thrombosis, the endothelial dysfunction occurs at the level of the microvascular bed (Franscini et al. 2004). Therefore, it would seem more appropriate to use capillary ECs isolated from the affected organ for its molecular events contributing to the disease rather than the non-specific analysis of the HUVECs under the local pathological environments. In the pathogenesis of osteonecrosis of femoral head (ONFH), there are evidences that a defect in endothelial cells located in the feeding vessels of the femoral head called Regional Endothelium Dysfunction (RED) plays a crucial role in the initiation and progression of

the disease (Kerachian et al. 2006). In some vascular diseases, the activation of the endothelium in response to inflammatory stimuli, such as interleukin 1β or tumor necrosis factor-ß dysregulates the endothelium function. This dysregulation leads to microvascular thrombosis, organ damage, multiple organ dysfunction, and often death of the affected tissue (Franscini et al. 2004). Occlusion of arteries and decreased vascularity in bone tissue has been involved in the pathogenesis of disease states such as osteonecrosis (Atsumi and Kuroki 1992). We have recently proposed that alteration in the vascular system with a pivotal role for ECs could lead to ONFH (Kerachian et al. 2006). Isolation of the ECs from human bone tissue to permit the study of the molecular and/or genetic abnormalities potentially involved in bone disease however, has not yet been described. In this study, we isolated and confirmed the presence of ECs from femoral cancellous bone tissue. These findings could facilitate the study of the potential roles of regional ECs in bone diseases such as ONFH and osteosarcoma.

Materials and Methods

Cell culture

HUVECs (Human Umbilical Vein Endothelial Cells) and HSCs (Human Stromal Cells) were obtained from Cambrex (Walkersville, MD) and American Type Culture Collection (Manassas, VA), respectively. The HUVECs were cultured in EBM-2 media (Cambrex, Walkersville, MD) with the microvascular additives (supplemented with human endothelial growth factor, hydrocortisone, fetal bovine serum (2%), vascular endothelial growth factor, human fibroblast growth factor type B, R3 insulin-like growth factor 1, ascorbic acid, heparin, gentamicin and amphotericin B, according to the manufacturer's protocol Cambrex, Walkersville, MD). HSCs were cultivated in DMEM (Dulbecco's modification of Eagles medium) supplemented with 10% FBS (Montreal Biotech Inc. Kirkland, PQ, Canada), 100 u/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B.

Isolation of human bone-derived endothelial cells (hBDECs)

The femoral heads or bone fragments from femoral heads were obtained from patients with an average age of 65 years old undergoing surgery of the hip joint after informed consent was obtained. An institutional review board approved the study and all study procedures were in compliance with institutional guidelines. Cancellous bone from the subchondral region of the femoral head was aseptically collected and rinsed on ice-cold DMEM supplemented with 10% FBS, 100 u/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin. The hBDECs were isolated using a modified technique previously described by Zhang et al. for the isolation of EC from mice bone tissue (Zhang et al. 1998). Bone was washed twice with 2.5% FBS in PBS (Phosphate Buffer Saline), and centrifuged at 300 x g for 3 minutes at 4°C. The pellet was treated with ice-cold Hanks` Balanced Salt Solution, (without calcium and magnesium) supplemented with 0.2% type I collagenase (Sigma, St. Louis, MO) for 1 hour at 37°C, with gentle shaking. After digestion, the solid tissue was re-suspended in 2.5% FBS/PBS, filtered through a 70 µM mesh cell strainer (Falcon, Becton Dickinson), and washed with 2.5% FBS/PBS. The

cells were counted using trypan blue exclusion for evaluation of viability. Cells were re-suspended in EBM-2 media with the recommended microvascular additives in EBM-2 media and seeded into fibronectin-coated dishes (Biocoat, Becton Dickinson) and incubated at 37°C with 5% CO_2. Culture medium was changed the following day, and then every other day.

After 3 weeks of culture of primary cells in EBM-2 media with the appropriate microvascular additives, the cells became almost confluent and they were harvested with 1X cell dissociation solution (Sigma, St Louis, MO) for 10 minutes at 37°C. The cells were washed with 2.5% FBS in PBS, resuspended in 2 mM EDTA in PBS, incubated for 15 minutes at room temperature, and then washed with FACS (fluorescence activated cell sorting) wash buffer (0.5% bovine serum albumin (BSA), 0.1% NaN_3, in PBS). Cells were incubated with R-Phycoerythrin (R-PE)-conjugated mouse anti-human monoclonal CD31 antibody (BD, Mississauga, ON, Canada) for 50 minutes on ice, followed by three washes with FACS wash buffer and incubation with anti-PE MicroBeads antibody (Miltenyi Biotec, Auburn, CA) for 15 minutes at room temperature. After preparing the separation columns, the magnetic separation was performed according to the manufacturer`s protocols, using an OctoMACS^{TM} separator (Miltenyi Biotec, Auburn, CA). To increase the purity of the positively selected cell population, positively selected cells were passed over a second immunomagnetic column. These positively selected cells and cells that did not attach to the first column ("depleted cells") were plated separately onto fibronectin-coated dishes.

Fluorescence Immunohistochemical (FIHC) study

Immunohistochemical methods were used to characterize the phenotype of hBDECs. Positive and depletion cells purified as described above were plated onto Lab-tek chamber slides (Nunc. Naperville, IL) coated with 1% gelatin and grown to near confluence. After three washes with PBS, cells were fixed in acetone for 20 minutes at –20° C. The cells were incubated with an 8:100 dilution of mouse anti-human CD31 monoclonal antibody (BD, Mississunga, ON, Canada) for 1 hour at 4°C. After three times washing the slides with PBS (5 minutes for each step) the anti-CD31 antibodies were

detected by incubation with a 1:200 dilution of Alexa Flour® 568 goat anti-mouse IgG_1 (Molecular Probes, Invitrogen Detection Technologies, Eugene, Oregon, USA) for 1 hour at 4°C. At the same time, the cells were also incubated with 1:100 dilution of sheep anti-human von Willebrand Factor antibody conjugated with fluorescein isothiocyanate (FITC, Serotec, Raleigh, NC, USA). Then, the cells were stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma) at 0.3 nM at room temperature for 5 minutes. Control cells were incubated with washing buffer in place of primary antibody. A fluorescence microscope (Olympus BX60F5, Olympus Optical Co., Japan) was used for the examination.

Flow cytometry analysis of antigen expressed in fresh and cultured primary cells

The isolated cells were characterized using flow cytometry analysis for the determination of cell surface immunophenotype. All experiments were carried out using cultured primary cell in passages 0 to 5.

Freshly isolated primary cells. After the mechanical and enzymatic isolation steps, red cell lysis was performed using PharMLyse (PharMingen, Becton Dickinson) according to the manufacturer's recommendations. Cells were then centrifuged at 200g for 5 minutes at 4°C. Pellets were washed with 2.5% FBS/PBS and re-suspended in 2mM EDTA/PBS for 15 minutes at room temperature. The cells were washed with FACS wash buffer.

Cultured primary cells. After two weeks of culture in EBM-2 media with the appropriate microvascular additives, primary cells were harvested following 5 minutes incubation with 0.05% trypsin and 0.53 mM EDTA (Wisent Inc., Saint-Jean-Baptiste de Rouville, PQ, Canada) or 1X cell dissociation solution for 10 minutes at 37°C. The cells were washed with 2.5% FBS in PBS and re-suspended in 2 mM EDTA in PBS and incubated for 15 minutes at room temperature, and then washed with FACS wash buffer.

Cells were incubated with primary antibody for 30 minutes at room temperature, followed by three washes with FACS wash buffer. When appropriate, cells were incubated with a secondary antibody for 30 minutes at room temperature and washed three times with the FACS wash buffer. Controls were treated with secondary antibody alone. Primary antibodies were as follow: R-Phycoerythrin (R-PE)-conjugated mouse anti-human monoclonal CD31 antibody (BD, Mississauga, ON, Canada), mouse monoclonal antibody

to human CD105 (Endoglin) conjugated with FITC (ID Labs inc., London, ON, Canada), mouse anti-human vascular endothelial (VE)-cadherin monoclonal antibody (Chemicon, Temecula, CA), monoclonal antibody to Von Willebrand Factor (Immunotech, Marseille, France). Secondary antibodies were as follow: multiple absorption allophycocyanin (APC)-conjugated goat anti-mouse immunoglobulin specific polyclonal antibody (BD Mississauga, ON, Canada), APC-conjugated rat anti-mouse IgG_1 monoclonal antibody (BD, Mississauga, ON, Canada), FITC-conjugated rat anti-mouse IgG_{2a} monoclonal antibody (BD, Mississauga, ON, Canada). Fluorescence data were collected using a Becton Dickinson FACScan (FACSCalibur, BD).

Morphological examination of hBDECs cultured on matrigel

Matrigel (Becton Dickinson) was diluted with cold DMEM/10% FBS at a concentration of 10 mg/ml and 1 ml of this dilution was applied to each well of a 24-well plate. hBDECs and HUVECs were cultured on matrigel-coated wells in EBM-2 media with the appropriate microvascular additives. HSCs were also cultured on matrigel in DMEM/ 10% FBS. HUVECs and HSCs were used as positive and negative controls, respectively, for the identification of endothelial cell phenotype. The specimens were viewed using a phase contrast microscope (Olympus) at 8, 24 and 48 h after culturing.

Results

Isolation and characterization of hBDECs

hBDECs were isolated from femoral bone tissue at subchondral region. Initially, the primary cells isolated by enzymatic and mechanical procedures were cultured in appropriate medium on fibronectin-coated dishes. After 2 hours of incubation, the primary cells began to attach, and underwent cellular division. Two days later the morphology of primary cells was compared with that of HUVECs. Initially, all the cultured cells displayed a polygonal morphology similar to HUVECs. However, cells with "spindle cell" morphology often emerged and as such, became the prominent cell type in culture over time. To characterize the cultured cells, analysis included the expression of human CD105 (Endoglin), CD31 (PECAM-1), vWF and VE-Cadherin antigens, all standard markers of EC phenotype, using flow cytometry. Table 1 summarizes the comparison of cell surface protein expression on primary cells with HUVECs and HSCs in culture. As shown in Table 1, there is a steady decrease in expression of CD31 antigen in cultured cells.

Subsequently, in order to obtain a purified hBDECs population, the cultured isolated cells were sorted using immunomagnetic beads. Immunomagnetic bead separation yielded two morphologically distinct purified populations of primary cells, polygonal and spindle shape (Figure 1). The polygonal cells were MicroBeads positive cells as anti-CD31 antibodies reacted strongly with those cells, whereas the spindle-shaped cells showed no interaction with the MicroBeads demonstrating a lack of expression of the CD31 antigen. The MicroBead positive cells (CD31 positive cells) displayed a polygonal morphology similar to HUVECs in culture and grew in a "cobble stone" pattern. The polygonal cells showed expression of vWF and CD31 antigens in the FIHC study (Figure 2).

When hBDECs were cultivated on matrigel, they were able to spontaneously reorganize in cord-like structures after several hours, similar to HUVECs. In contrast, HSCs were not able to form a tubular pattern on the matrigel. The hBDECs started to migrate to the inside of the lumen after 48 hours (Figure 3) and they had a stronger ability to form a tubular pattern than HUVECs (data not shown).

Table 1- Comparison of numbers of cells expressing cell surface antigens of hBDEC, HUVEC and HSC.

Marker	**HUVEC**	**HSC**	*HBDEC*	
			In fresh primary cells	**In cultured cells**
CD105$^+$	100%	95.2%	89.7%	38.7%‡
CD31$^+$	99.7%	0.5%	28.4%	1.6%†, 0.7%‡, 0.3%§
CD105$^+$ CD31$^+$	99.9%	0.7%	25.1%	0.3%‡
VWF$^+$	81.8%	0.3%	N.D.	35.8%‡
VE-Cad+	62.3%	0.0%	N.D.	12.5%‡

†1 week, ‡2 weeks and §4 weeks of culture.

Abbreviations: HUVECs: Human Umbilical Vein Endothelial Cells, HSC: Human Stromal Cells, hBDEC: human bone derived endothelial cell, vWF: Von Willebrand's Factor, VE-cad: vascular endothelial (VE)-cadherin

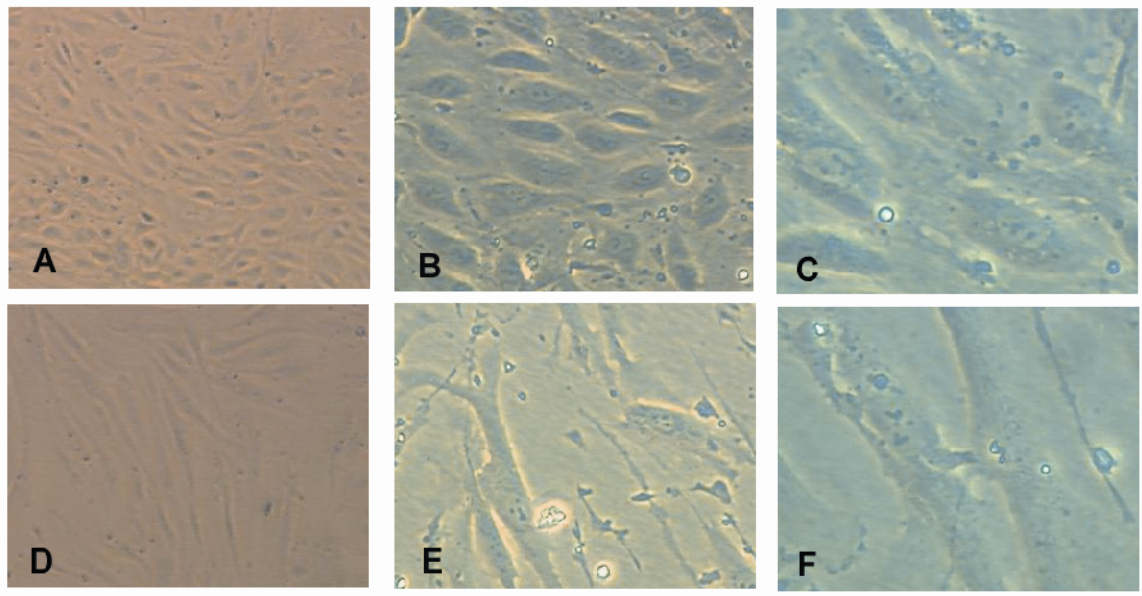

Figure 1. Phase-contrast micrograph of CD31-immunomagnetic bead separated cultured primary cells. Two morphologically different populations of cells (A, B &C: polygonal cells and D, E & F: spindle-shaped cells) obtained by microbead separation from passage three (week-4) cultured primary cells (magnification A, D x40, B, E x100 & C, F x200).

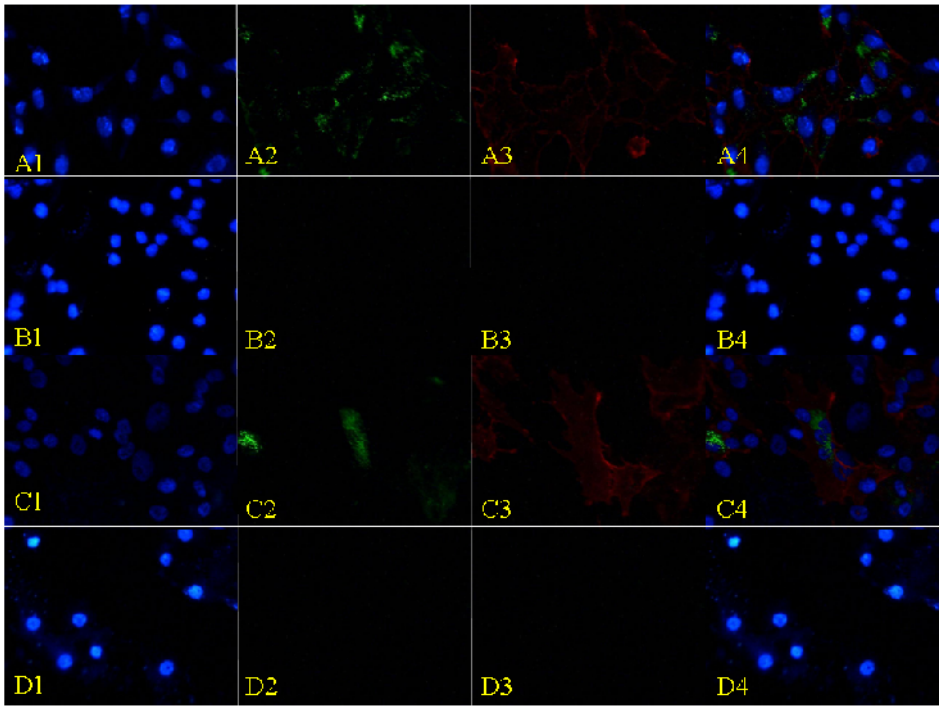

Figure 2. Immunofluorescent characterization of hBDECs purified with anti-CD31-beads. The expression of von Willebrand Factor in cytoplasm (green) and CD31 on membrane (red) shown in HUVECs (A1-4) and hBDECs (C1-4). The nuclei are stained with DAPI (blue). The hBDECs were grown in culture in passage 2. The microbead depleted cells and HSCs are shown in B1-4 and D1-4, respectively (magnification x200).

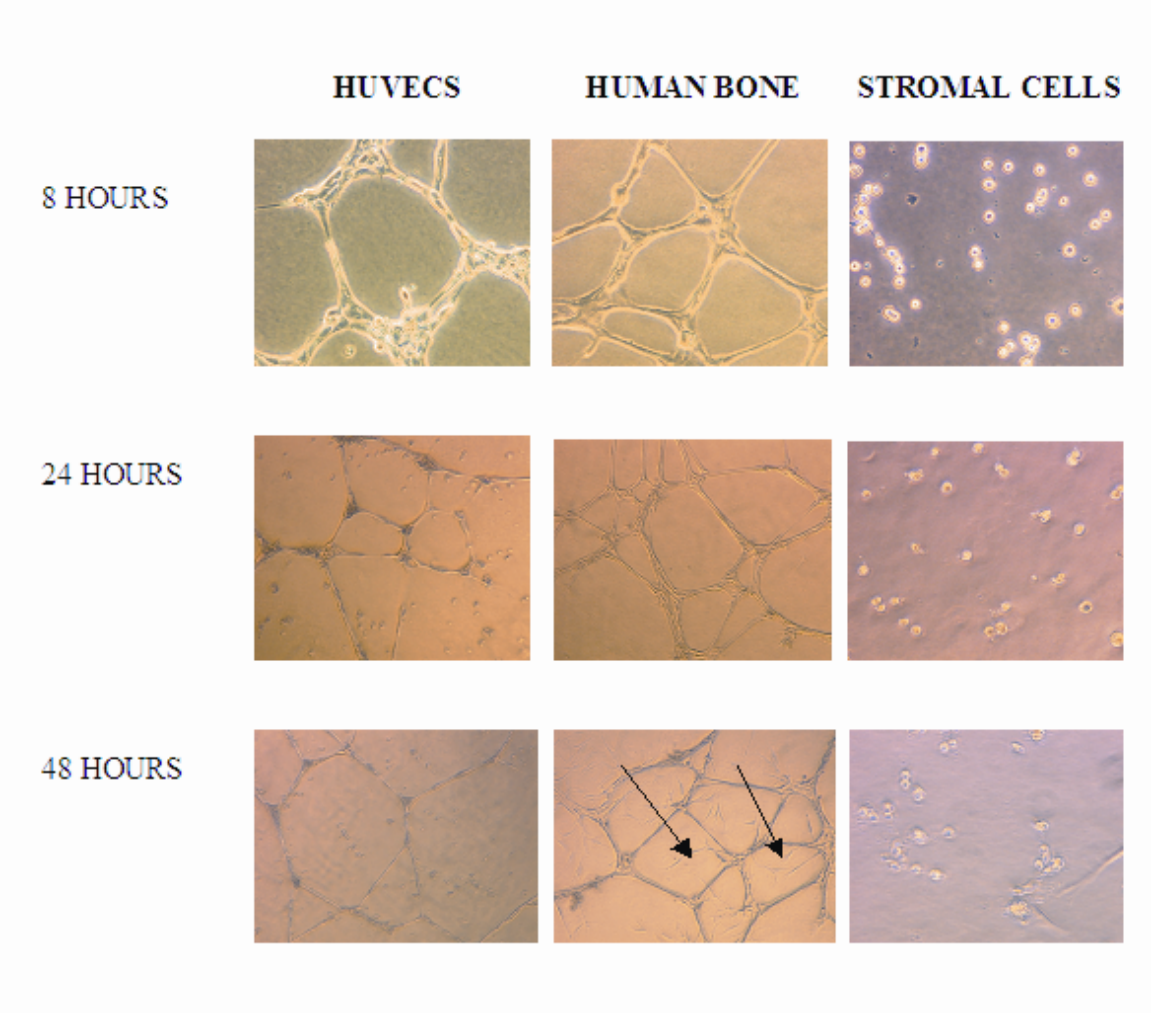

Figure 3. Matrigel assay: human bone-derived primary cells forming tubular structures in matrigel after 8 hours. Cells started to migrate to the inside of the lumens after 48 hours (arrows).

Discussion

Several studies have reported the role of tissue-specific characteristics of ECs in the pathogenesis of various disorders (Madden et al. 2004; Patel et al. 2003; Rafiee et al. 2003; St Croix et al. 2000). Heterogeneity of ECs according to tissue-specific vascular-bed haemostasis has become an accepted concept in vascular biology (Aird 2003; Paunescu et al. 2003; Garlanda and Dejana 1997). Hence, it seems to be more appropriate to study specifically ECs isolated from the affected tissue in a vascular disease state rather than using primary endothelial cells from another origin, such as HUVECs, or an EC line. Several reports have shown vascular thrombosis in ONFH (Atsumi and Kuroki 1992; Glueck, Freiberg, and Wang 2003; Jones, Jr. 1992), and it is likely that the bone endothelium locally participates in the formation of the thrombus (Kerachian et al. 2006). It is also known that the ECs play a central role in neovascularization, which participate in tumor formation in cancer such as osteosarcoma (Cai et al. 2004; Mikulic et al. 2004) or Ewing's sarcoma (Bolontrade, Zhou, and Kleinerman 2002).

In this report, we isolated and confirmed the presence of ECs coating the sinusoids and vessels extended to the subchondral region of the femoral head in humans. Several studies have shown that ECs could play a pivotal role in microvascular thrombosis located in the subchondral region of the femoral head in ONFH (Kerachian et al. 2006).

Recently, several methods have become available for the isolation and purification of ECs from non-bone tissue, using beads coated with antibodies to Ulex europreus agglutinin-1 (Christenson and Stouffer 1996; Hewett and Murray 1993; Madden et al. 2004; Masek and Sweetenham 1994; Matsubara et al. 2000; Quirici et al. 2001; Rafii et al. 1994; Wu et al. 2004), CD31 (Hewett and Murray 1993; Matsubara et al. 2000; Wu, Hofman, and Zlokovic 2003), P1H12 (Madden et al. 2004; Parker et al. 2004; St Croix et al. 2000), E-selectin (Richard, Velasco, and Detmar 1998) or CD34 (Asahara et al. 1997; Eggermann et al. 2003). Although ECs have been isolated from human bone marrow (Masek and Sweetenham 1994), this study is the first report on the isolation and confirmation of endothelial cells from human cancellous bone tissue.

In this study, based on the flow cytometry analysis of freshly isolated primary cells, only 28% of the cells that expressed CD105 antigen also expressed CD31 antigen. Although CD105 antigen is highly expressed on human vascular endothelial cells (Burrows et al. 1995), its expression detected in other cell types include activated monocytes, fibroblasts, differentiated macrophages, erythroid precursors, vascular smooth muscle cells, melanocytes, mesangial cells, heart mesenchymal cells, follicular dendritic cells and syncytiotrophoblast (Fonsatti and Maio 2004). CD31 antigen, on the other hand, is constitutively expressed on the surface of all endothelium and is not present on any other cell type outside the white blood cell population (Hewett and Murray 1993). As a result, it is more likely that cells expressing both CD105 and CD31 antigens, are in fact, endothelial cells.

When the isolated cells were cultured on fibronectin or gelatin coated dishes, initially, all cells displayed a polygonal morphology similar to HUVECs. However with time, spindle type cells emerged and overwhelmed the culture. The cultured cells in this study were found to be positive for endoglin (CD105), and to a lesser extent, for von Willebrand Factor and VE-cadherin, two well recognized markers for ECs (Garlanda and Dejana 1997; Petzelbauer, Halama, and Groger 2000; Schmeisser and Strasser 2002). Although the expression of CD31 was significant on freshly isolated cells, its level is much lower in cultured cells and gradually decreases to the level of becoming almost non detectable upon prolonged culture. The emergence of the spindle shape cells (which do not express CD31 antigen) in the prolonged cell culture may explain the loss of CD31 expressing cells. To assure the purity of the endothelial cell population in our isolation procedure, we performed immunomagnetic bead separation using anti-CD31 antibodies conjugated beads. The CD31 antibody purified cells (positively selected cells) were found to be polygonal in shape and they formed a ‘cobble stone” morphology characteristic of endothelial cell growth *in vitro* (Hewett and Murray 1993). The depleted cells, however, displayed spindle shape morphology in culture. Masek et al showed that the expression of CD31 was associated with a polygonal cell morphology isolated from human bone marrow in comparison to the spindle-shaped cells (Masek and Sweetenham 1994). The endothelial origin of the polygonal cells was indicated by their expression of CD31 and vWF, which is generally considered to be one of the most specific markers for endothelial

cells (Garlanda and Dejana 1997). It has been demonstrated that endothelial cells cultured *in vitro* will form capillary-like tubules when exposed to ECM (extracellular matrix) protein, a typical although not specific behavior of cultured endothelial cells. Matrigel matrix, which contains a mixture of ECM proteins, mainly laminin and collagen IV but also entactin, as well as a mixture of growth factors, has been used *in vitro* angiogenesis functional assays to characterize endothelial cells (Wu, Hofman, and Zlokovic 2003). We observed that the hBDECs obtained with our procedure, either freshly isolated or maintained in culture, also formed a capillary tubular pattern (Figure 3), which strongly supports the hypothesis that these are of endothelial origin.

In summary, we isolated and confirmed the presence of endothelial cells from human cancellous bone tissue. The cells isolated were characterized and confirmed to be of endothelial origin by the expression of specific endothelial cell surface markers and by the functional assay revealing cellular ability to form tubules. Although in this study hBDECs have been isolated from the cancellous bone tissue of the femoral head, this technique can be expanded to isolate ECs from other human bone tissues. The cells isolated could provide a source of primary population of endothelial cells at the disease site for the biological analysis of human vascular bone diseases such as ONFH and osteosarcoma.

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Amendments:

Cultured primary cells. After two weeks of culture in EBM-2 media with the appropriate microvascular additives, primary cells were harvested following 5 minutes incubation with 0.05% trypsin and 0.53 mM EDTA (Wisent Inc., Saint-Jean-Baptiste de Rouville, PQ, Canada). We had determined trypsin-sensitivity of each antigen detected on primary cells. For the primary cells targeting their CD31 antigen 1X cell dissociation (protease free) solution for 10 minutes at 37°C was used. The cells were washed with 2.5% FBS in PBS and re-suspended in 2 mM EDTA in PBS and incubated for 15 minutes at room temperature, and then washed with FACS wash buffer.

The legend of figure 3 is modified to:

Figure 3. Matrigel assay: human bone-derived primary cells forming tubular structures in matrigel after 8 hours. Cells started to migrate after 48 hours (arrows).

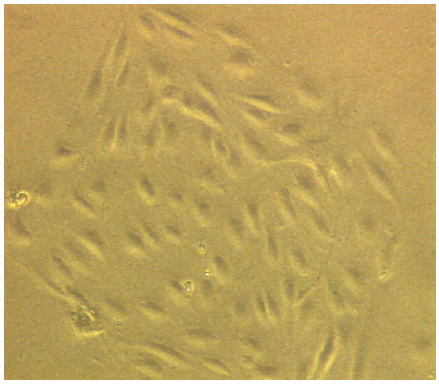
Discussion

… In this report, we isolated and confirmed the presence of ECs coating the vessels extended to the subchondral region of the femoral head in humans…

Chapter 6

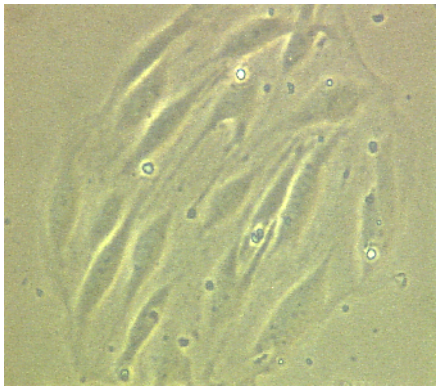
Similarly to the previous chapter describing the isolation of human endothelial cells from the femoral head, we isolated endothelial cells from rat femoral head bone tissue using the method described from our paper (18) (Figure 1). The bone-derived endothelial isolation method confirmed that endothelial cells are part of the main architecture of the femoral head.

Also, given that expansion of human endothelial cells in culture could affect the profile of gene expression, we have used a method involving a double positive selection using immunomagnetic beads to achieve a higher degree of purity for the targeted cells (Figure 2). Anti-human CD 31 (PECAM-1) and anti-human CD 105 (endoglin) microbeads were thus used to target human endothelial cells. This method was designed and optimized using 2% human umbilical venous endothelial cells (HUVEC) mixed with human fibroblast cells. Although endothelial cells were isolated with a purity of 90-95% in these experiments, endothelial cells were isolated in very small numbers, insufficient for gene profiling studies from human necrotic tissues with this method. Consequently, our approach has been to proceed with gene profiling analysis using the total cell population isolated from the femoral head in the rat model. For this purpose, we selected two strains of rats, Wistar Kyoto and Fisher rats, which were respectively susceptible and resistant to develop ANFH. In the next manuscript, we evaluated the gene expression pattern of cells located in the femoral head of Wistar Kyoto rats. To avoid interference by inter-strain genetic variabilities unrelated to ANFH, gene expression comparison was performed in only one strain (Wistar Kyoto), comparing steroid and placebo-treated rats. Comparing transcription profiles of steroid and placebo-treated rats would reveal the effect of steroid and ANFH on gene expression.



100X

Fig. 1: Endothelial cells isolated from rat femoral head bone tissue.



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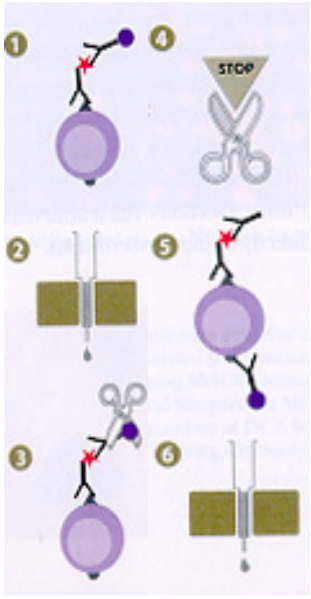

Fig. 2: Schematic figure of a double positive selection. 1. Antibody conjugated to bead binds to anti-CD 31 antibody recognizing target cells, 2. Cells trapped in the column, 3. Bead released from its antibody using cutting enzyme, 4. Inactivation of enzyme, 5. Binding anti-CD 105 antibody bead-conjugated to target cells, 6. Endothelial cells trapped in column (Miltenyi Biotec double positive selection manual).

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Microarray Analysis of Gene Expression in Glucocorticoid-Induced Avascular Necrosis using a Rat Model: New Insights in the Pathogenesis

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Abstract

Rationale: Avascular necrosis of the femoral head (ANFH) occurs as a result of interruption to blood flow at the level of the microcirculation. Although ANFH is frequently seen in several conditions however we are unable to predict who will develop the disease and we currently have little understanding of its pathophysiology. It is well known that ANFH tends to develop after exposure to corticosteroids, one of the major risk factors for this condition, and that microvascular thrombosis is a central phenomenon in ANFH development. As systemic thrombophilia has been recently found to have a weak correlation with the development of ANFH, it is hypothesized that the documented microvascular thrombosis is related to endothelial cell dysfunction, diffuse femoral head cellular apoptosis (endothelial cell, osteoblast, osteocyte) and subsequent bone necrosis.

Methods: Here we tested the hypothesis that corticosteroids contribute to a differential gene expression in the femoral head of rats for early ANFH. Forty Wistar Kyoto 5 week-old rats were randomized to either a treatment group (24 rats implanted with prednisone pellets, average dose of 1.5mg/kg/day) or an age-matched control group (16 rats implanted with placebo pellets) for a period of 6 months. At 6 months the rats were sacrificed. Histological analysis and TUNEL assay were performed to test for ANFH. Total mRNA was extracted from femoral heads of both treated and placebo rats. Affymetrix exon array was performed on 15 selected RNA samples from three different groups based on the histological results: Group 1 (steroid-, ANFH-), Group 2 (steroid+, ANFH+), Group 3 (steroid+, ANFH-). Comparisons of interest of gene expression profiles were Group 2 vs 1 and Group 3 vs 2. RT-PCR analysis and fluorescence immunohistochemistry (FIHC) studies were performed as validation methods.

Results: Rat exon array demonstrated a significant upregulation of a various number of specific genes, particularly alpha-2-macroglobulin, when comparing steroid-treated Wistar Kyoto rats that had developed ANFH (G2) vs placebo-treated rats (G1). These results were validated by RT-PCR and by FIHC. This particular gene of interest has haemostatic, osteogenic and cartilage functions – particularly relevant for ANFH.

Conclusion: Together, these findings suggest that corticosteroid-induced ANFH in rats might in part be mediated by the regulation of a specific gene, alpha-2-macroglobulin,

sharing haemostatic, osteogenic and cartilage properties. More studies are needed to determine specifically at which level of the endothelial cell activation and/or apoptosis pathway the effect of this gene is modulated in corticosteroid-treated rats that develop ANFH.

Keywords: osteonecrosis, avascular necrosis, alpha-2-macroglobulin, Affymetrix, microarray

Introduction

Atraumatic avascular necrosis (AVN) of bone usually occurs in areas with limited collateral circulation. The process is precipitated when the blood supply to the bone is disrupted. Commonly involved bones include talus, scaphoid [1], humerus [1,2], mandible [3,4] and the femoral head-which is the most frequently affected bone area [5]. Avascular necrosis of the femoral head (ANFH) is a disabling and progressive condition in young patients, which if untreated, will lead to femoral head collapse requiring total hip replacement [6]. ANFH has been associated with various conditions such as trauma, glucocorticoid (GC) therapy, alcoholism, abnormal lipid metabolism [7], and diseases resulting in vasculitis [8]. Aside from traumatic conditions, the pathogenesis of osseous ischemia is not yet understood. The most notable unsolved issues concern the earliest pathogenic factors involved in the disease development. Two major limitations in the past have impeded the delineation of the pathophysiology of ANFH: 1) a comprehensive study of the interaction between the bone and its vascular supply, in particular the endothelial cells, had not been performed and 2) the lack of suitable animal models for this condition. While epidemiological studies and the introduction of magnetic resonance imaging have produced valuable insights into the diagnosis of ANFH, there is currently no standard method available to evaluate the activity status, bone turnover, and the prognosis of the disease [9]. Identifying high-risk individuals and quantifying their risk in terms of clinical and pathophysiological characteristics would be key to successful prevention and treatment. Besides the increased incidence in the various conditions and etiologies mentioned earlier, the fact that ANFH is sometimes seen in twins and in familial clusters suggests that genetic factors are involved [10,11]. New evidence of increased incidence of ANFH in specific animal models also provides further evidence of the existence of susceptibility genes [12]. Some thrombophilic mutations have been identified as risk factors for ANFH in Caucasians [13], but a coagulation-based genetic risk factor may not specifically provide an answer to its pathogenesis. Although the prevalence of coagulation abnormalities in patients with ANFH is increased in some

studies compared to controls [14-18], the vast majority of ANFH patients do not demonstrate consistently any known coagulation disorders [19,20].

Hence, the current pathophysiological model of ANFH puts forward a multiple hit theory in which, with the accumulation of risk factors ultimately resulting in ANFH [21]. Amongst the many risk factors, GCs play the leading role in ANFH [22].

In a previous *in vivo* pilot study, an inbred rat strain susceptible to develop glucocorticoid-induced avascular necrosis was identified. In the present randomized controlled study using this *in vivo* rat model, we employed gene profile analysis in susceptible rats in order to study the pathogenesis at an early stage of the disease. Because of the similarity of the human genome with rat, knowledge of gene expression pattern and the events that contribute to the genesis and progression of ANFH in this rat model could provide insights to a better understanding of the pathogenesis in humans.

Materials and methods

Experimental animals and their maintenance

Forty Wistar Kyoto rats (ages 4 weeks old) were purchased from Charles River Laboratories (Pointe-Claire, QC, Canada). The ears of all rats were tagged and they were housed in plastic cages (2 to 4 animals per cage) under standard laboratory conditions with a 12-hour dark/12-hour light cycle, a constant temperature of 20 °C, and humidity of 48%. Food and water were provided ad libitum with a standard rodent diet. The weight of the rats were followed weekly before and after the implant of the prednisone pellet for the first 3 consecutive weeks and then every month until the end of the experiment. All experiments were conducted under an animal protocol approved by the McGill Animal Care Department.

Glucocorticoid administration

Slow-release prednisone pellets (Innovative Research of America, Sarasota Florida, USA) were implanted subcutaneously to 24 Wistar Kyoto rats (12 males and 12 female rats) at the age of 5 weeks. Each pellet was implanted underneath the skin on the lateral side of the neck by surgically making an incision and a pocket of about 2 cm long beyond the incision site. The pellet was placed in the pocket and the incision was sutured. To maintain a constant dosage with the weight of the rats during the period of the experiment, second and third pellet implantations were performed using the same procedure at 2 and 3 months respectively. The average dose of prednisone was 1.5 mg/kg/day for a period of 6 months. For the control group, 16 age-matched Wistar Kyoto (8 males and 8 females) rats received placebo pellets (Innovative Research of America).

Histologic Examination

The rats were sacrificed with an overdose of ketamin/xylazine after 6 months of “treatment” at the age of 30 weeks. Tissue samples were obtained from the proximal femur containing the femoral head. Some samples were preserved put in RNALater

(QIAGEN Inc., Mississauga, ON, Canada) for later RNA extraction and some samples were fixed for histological examination. Bone samples were fixed in 10% neutral buffered formalin, then decalcified in 4% ethylenediamine tetraacetic acid (pH 7.2) (Sigma-Aldrich, St. Louis, MO, USA). The specimens were processed routinely and embedded in paraffin. Tissue sections were sectioned parasagitally with a rotary microtome at 4 to 5 microns thickness, stained with hematoxylin and eosin and evaluated by light microscopy.

Tissue samples were analyzed in a blinded fashion by two experienced bone pathologists (A.N. & L.R.B.). The histological findings of an established ANFH are generally defined as dead trabeculae exhibiting empty lacunae with or without appositional bone formation [23] as shown in figure 1. While the development of ANFH proceeded through various stages, it was essential for this study to detect the early as well as the late stages of the condition. With this objective in mind, we adopted the criteria of Arlet *et al* namely degeneration, necrosis, and disappearance of marrow cells as well as the nuclear disappearance and hypochromasia of trabecular osteocytes as early signs of ANFH [24]. Early signs of ANFH were also considered when apoptosis occurred in the osteocytes and osteoblasts (Fig. 2). Positivity for apoptosis was defined as more than three osteocytes and/or osteoblasts recognized in a high magnification field (x200) (Table 1).

Measurement of apoptosis in undecalcified bone section

Terminal dexoynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) was used to detect fragmented DNA known to be associated with apoptotic cell death. TUNEL assay on paraffin-embedded tissue sections was performed with the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA) as recommended by the manufacturer. Briefly, after deparaffinization and permibilizing the tissue sections with proteinase K, the slides were incubated with the reaction mixture containing recombinant TdT and biotinylated nucleotide for 1 hour at 37 °C inside a humidified chamber. Labeled DNA was visualized with horseradish-peroxidase-labeled streptavidin using 3,3′-diaminobenzidine (DAB) as the chromogen. DNase I-treated tissue sections were used as positive controls. Negative controls for the

study were sample slides processed using the same procedure but not treated with TdT Enzyme. All the slides were counterstained with 0.5% methyl green solution (0.5g ethyl violet (Sigma-Aldrich) in 100 ml sodium acetate buffer, 0.1M and pH.4.2), cleared, mounted and evaluated by light microscopy.

RNA extraction from rat bone specimens

Total RNA was extracted by an innovative method consisting of a combination of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini kit (QIAGEN Inc.) followed by DNase I treatment (QIAGEN Inc.). Briefly, femoral head specimens were removed from RNALater and washed thoroughly with DEPCI-treated PBS. Femoral head specimens were placed in liquid nitrogen. The specimens were ground to a fine powder with a porcelain mortar and pestle. TRIzol® 1 ml was then added to each ground femoral head specimen. After vortexing for 1 min, the homogenized specimen was incubated for 5 min at room temperature (RT) and 0.2 ml Chloroform (Sigma-Aldrich) was added per 1 ml of TRIzol®. After vortex use of 15 seconds the samples were incubated for 3 min at room temperature. The samples were then centrifuged at 12000 x g for 15 min at 4°C. The aqueous phase was removed from each sample and one volume of ethanol was added to it and mixed thoroughly. Up to 700 µl of the sample including any precipitate that may have formed was transferred into an RNeasy Mini Spin Column. The column was then processed according to the RNeasy Mini kit manufacturer instruction. Any Genomic DNA contamination was removed by treating the samples with DNase I. The RNA quality was assessed using RNA 6000 NanoChips with the Agilent 2100 Bioanalyzer (Agilent).

Affymetrix Exon Arrays

Affymetrix GeneChip® Rat Exon 1.0 ST array interrogating over 850,000 exon clusters within the known and predicted transcribed regions of the entire genome and about one millon probe sets was purchased. Affymetrix exon array was performed on 15 RNA samples of steroid-treated and non-treated rats divided in 3 groups based on histological evaluation: Group 1- Placebo/ANFH(-); Group 2- Steroid-treated/ANFH(+) and Group 3-

Steroid-treated/ANFH(-), each group consisting of 5 samples (Table 2). Biotin labeled targets for the microarray experiment were prepared using 1 µg of total RNA. Ribosomal RNA was removed with the RiboMinus Human/Mouse Transcriptome Isolation Kit (Invitrogen, Eugene, Oregon, USA) and cDNA was synthesized using the GeneChip WT (Whole Transcript) Sense Target Labeling and Control Reagents kit as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). The sense cDNA was then fragmented by uracil DNA glycosylase and apurinic/apyrimidic endonuclease-1 and biotin-labeled with terminal deoxynucleotidyl transferase using the GeneChip WT Terminal labeling kit (Affymetrix). Hybridization was performed using 5 micrograms of biotinylated target, which was incubated with the GeneChip Rat Exon 1.0 ST array (Affymetrix) at 45 ºC for 16–20 h. After hybridization, non-specifically bound material was removed by washing and specifically bound target was detected using the GeneChip Hybridization, Wash and Stain kit, and the GeneChip Fluidics Station 450 (Affymetrix). The arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix). Raw data was extracted from the scanned images and analyzed. We used Affymetrix Power tools (Affymetrix), R and in-house built Perl scripts to filter the background noise based on the "detection above background" results which is detection metric generated by comparing Perfect Match probes to a distribution of background probes. The data were normalized based on the Iter-PLIER algorithm by using Affymetrix Power tools, R and in-house built Perl scripts. The genes with low signal (less than 100) were removed from our study (software: in house built Perl script). The differentially expressed genes, between two groups (G2 vs G1 and G3 vs G2), were detected using in house built R script, infer with *t*-test and adjusted with *Benjamini and Hochberg FDR* method [$p<0.05$, Fold Change (FC)>1.5].

Real-Time Polymerase Chain Reaction (SybrGreen RT-PCR)

Real-time PCR was carried out according to the protocol provided by the manufacturer for the QuantiTect SYBR®Green RT-PCR kit (QIAGEN Inc.). QuantiTect Primer Assays (Rn_Has2_1_SG, Rn_Col2a1_1_SG, Rn_Mia1_1_SG, Rn_Pfn2_1_SG, Rn_Ccl9_1_SG, Rn_Actb_1_SG, Rn_A2M_1_SG) were provided by QIAGEN Inc. and a thermal cycler (Prism 7900, Applied Biosystems, Foster City, CA, USA) was used. The reaction was set

up in 10 µl final volume applying the following conditions: cycling 50 ºC (30 min), 95 ºC (15 min) and for 45 cycles the conditions were 94 ºC (15 Sec), 55 ºC (30 Sec) and 72 ºC (30 Sec). For the relative quantification of gene expression, the comparative threshold cycle (ΔCt) method was employed and normalized against β-Actin rRNA, which was measured by the same method. All PCR reactions were performed in triplicate. Control reactions were set up lacking reverse transcriptase to assess the level of contaminating genomic DNA.

Fluorescence Immunohistochemical (FIHC) study

Paraffin-embedded sections were placed at 60 °C for 15 min, incubated in xylene for 15 min, and then transferred sequentially into 100% ethanol, 95% ethanol, 70% ethanol, and 50% ethanol for 5 min at RT. Sections were rinsed in deionized water and the endogenous peroxidase activity was blocked with incubating sections in 3% H_2O_2 in distilled water for 5 min. The slides were washed in several changes of distilled water. Antigen was retrieved by incubating the slides in Digest-All^{TM} 3 (Invitrogen Immunodetection, CA, USA) for 10 min. After several washes with PBS the slides were stained using R.T.U. Vectastain® Universal Quick kit (Vector Laboratories, Inc., CA, USA) according to the manufacturer's instructions. Several primary antibodies were used: 1:200 dilution of mouse anti-rat α-2-macroglobulin globulin monoclonal antibody (clone 129736, R&D Systems, Minneapolis, MN, USA); prediluted mouse anti-rat collagen II antibody monoclonal antibody (Abcam Inc., Cambridge, MA, USA) or 1:50 dilution of rabbit anti-rat melanoma inhibitory activity (MIA) polyclonal antibody (Santa Cruz, Biotechnology, Santa Cruz, CA, USA). The slides were counterstained with 0.5% methyl green solution as described before.

Statistical Analysis

Data reported on microarray results were by using in-house built Perl scripts with t-test and adjusted with B-H FDR method, the differentially expressed genes were detected between two groups [$p<0.05$, Fold Change (FC)>1.5]. RT-PCR results were given as the mean ± SEM. Comparison between groups was made with Student's t-test. For small size samples Mann-Whitney U test was used since normal distribution of data was not

assumed. Differences were considered significant at P values less than 0.05. Principle compartment analysis (PCA) was performed using R package to provide a global view of how the various sample groups were related.

Results

Histological findings

The use of the TUNEL assay to detect apoptosis proved to be very informative. Apoptotic osteocytes were located in the osteonecrotic samples without features of inflammation and visible necrosis, such as hyperemia, round cell infiltration, or lipid cyst formation. There was no appositional bone formation associated with granulation tissue around dead bone in keeping with the early stages of avascular necrosis (osteonecrosis) (Fig. 2). When the same TUNEL reaction was performed on control tissue (without prior digestion with DNase), a fewer number of cells (one or two) were labeled.

Table 1: Histological findings of avascular necrosis of the femoral head (ANFH) in Wistar Kyoto rats

Sex	Treatment	No. of rats	OA/GC	OEL	EO	LO
Male	Placebo	6	2	1	1	1
Male	Prednisone	7	5	1	4	1
Female	Placebo	5	1	0	1	0
Female	Prednisone	12	3	2	1	2

OA/GC: osteocyte apoptosis and/or ghost cell, OEL: osteocyte empty lacunae, EO: number of early stages of osteonecrosis, LO: number of later stages of osteonecrosis

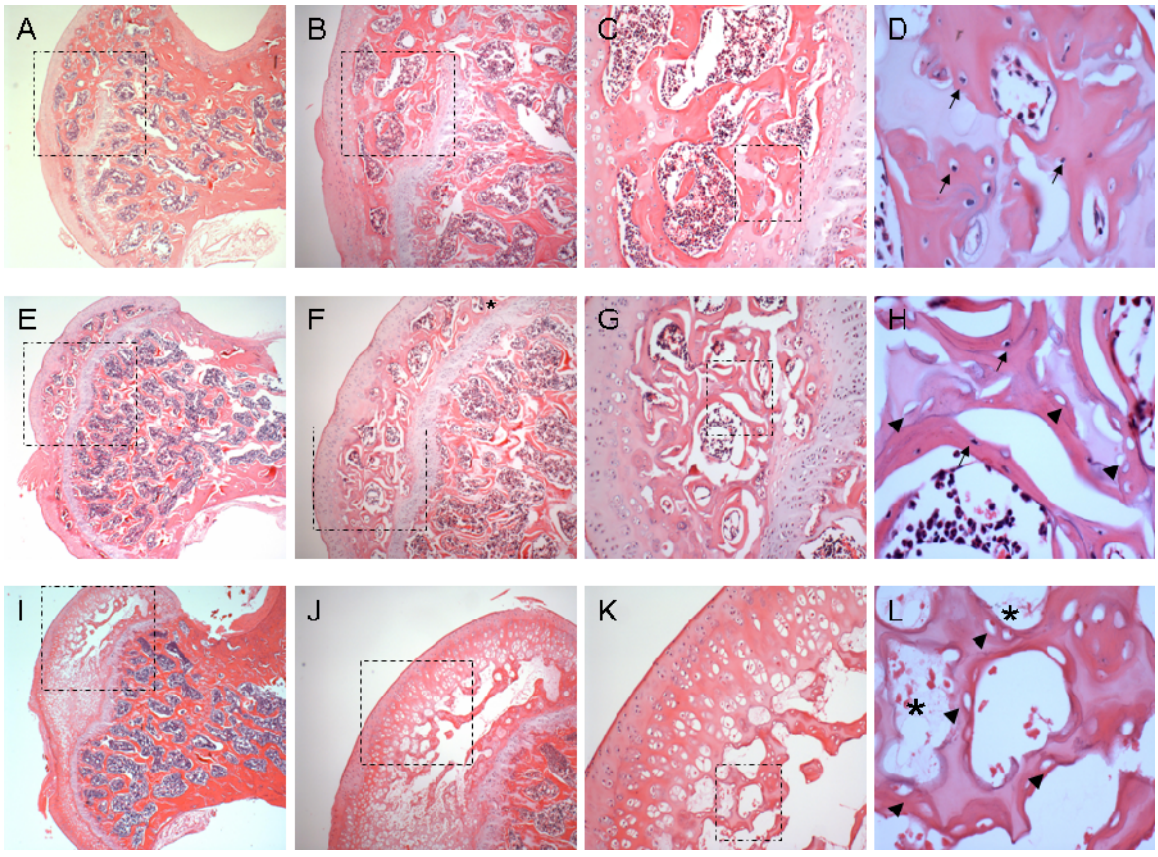

Figure 1. Photomicrographs showing histological findings in placebo- (A-D) and steroid-treated WKY rats (E-H & I-L) femoral heads. A-D: no osteonecrosis, normal osteocytes (arrow), **E-H**: early stage of osteonecrosis, normal osteocytes (arrow), empty lacunae (arrow head), **I-L**: late stage of osteonecrosis, empty lacunae (arrow head), complete necrosis of bone marrow (asterisk), H&E staining, **A**, **E**, **I** x20; **B**, **F**, **J** x40; **C**, **G**, **K** x100; **D**, **H**, **L** x200, dotted square chosen to be magnified.

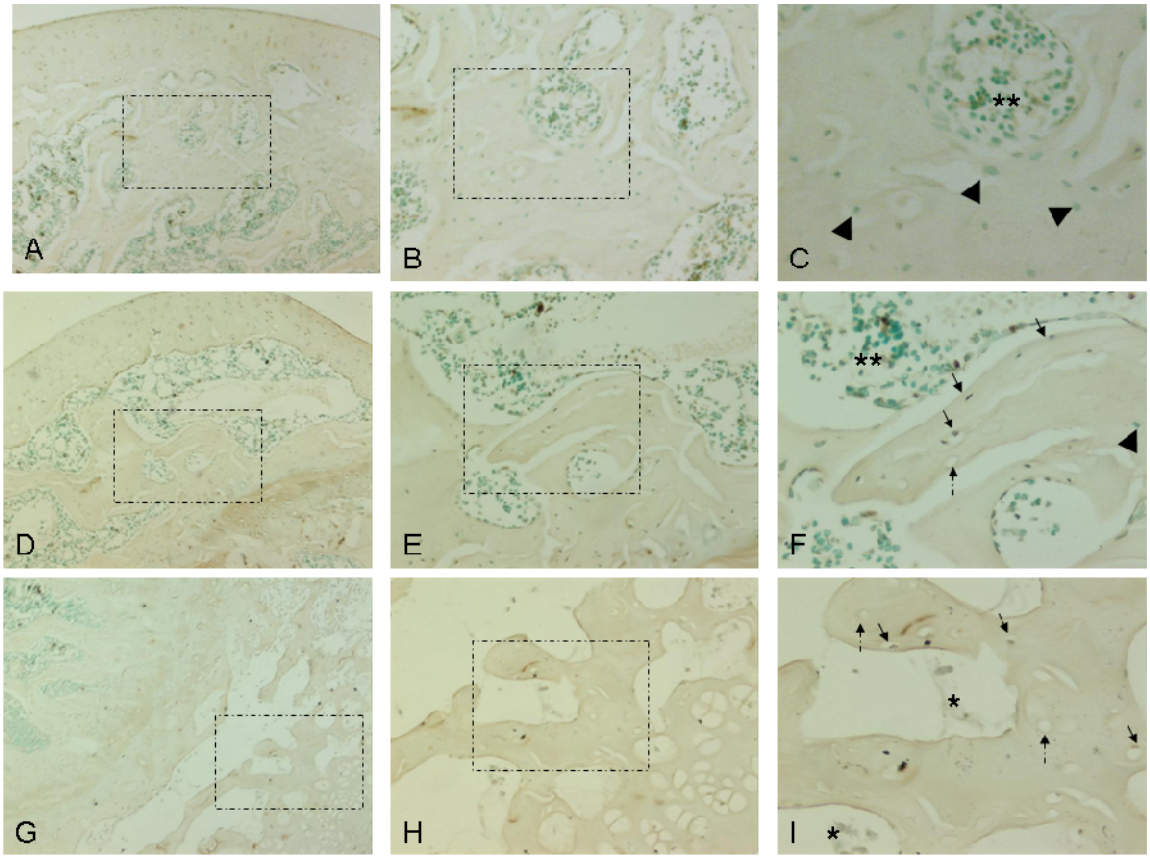

Figure 2. Apoptosis of osteocytes as a marker of early ANFH. TUNEL staining apoptosis assay counterstained with 0.5% methyl green solution. **A-C:** normal femoral head tissues in placebo-treated WKY rats, normal osteocytes (arrow head), normal bone marrow (double asterisk), **D-F**: early stage of osteonecrosis in steroid-treated WKY rats, TUNEL positive osteocytes (arrow), empty lacunae (dotted arrow), normal osteocytes (arrow head), normal bone marrow (double asterisk), **G-I**: late stage of osteonecrosis in steroid-treated WKY rats, TUNEL positive osteocytes (arrow), empty lacunae (dotted arrow), complete necrotic bone marrow (asterisk), **A**, **D**, **G** x40; **B**, **E**, **H** x100; **C**, **F**, **I** x200, dotted square chosen to be magnified.

Microarray analysis

We applied principle component analysis (PCA) to the genetic profiles of the three groups of rats. PCA is a useful technique to find patterns in gene expression data from microarray experiments. It is an effective method for reducing high dimensionality of data sets in order to create a two- or three-dimensional plot and capture variations in gene expression values [25]. PCA for the 15 samples showed that samples were organized into distinct groups (Fig. 3), which was correlated with the histological classification. Treatment with prednisone divided the rats into G1 versus the combined G2 and G3; the treated group had also been subdivided to two distinct subgroups (G2 versus G3). Group differences were considerably more important than interindividual differences and basically, there were no significant differences among the replicates in each group (Fig. 3).

In our Affymetrix analysis, we proceeded with two comparisons of interest: G2 versus G1 and G3 versus G2. G2 replicates were compared to evaluate GeneChip consistency and then compared with G1 and G3, separately, to generate a list of differentially expressed genes. As described in materials and methods section, these results were analyzed by a defined set of criteria in which the altered expression of a gene must have at least a change of ± 1.5 fold and a p-value less than 0.05. These criteria resulted in the identification of 51 and 6 genes with significant modulation in G2 compared with G1 and G3 to G2, respectively. For brevity, the selected set of genes related to the comparison of G2 versus G1 and, G3 versus G2 are presented in table 3 and 4, respectively. We categorized the significantly modulated genes according to their biological functions using DAVID, a functional annotation tool provided by National Institute of Allergy and Infectious Diseases, NIH. Modulated genes in the comparison of G2 and G1 were grouped mainly into clusters of skeletal development, ossification and bone remodeling classifications. According to our classification, there were changes in proteinaceous extracellular matrix. Other functional classes significantly represented in the steroid-induced avascular necrosis included response to steroid stimulus response, apoptosis, blood vessel morphogenesis, vasculature development, cell growth, proliferation and differentiation associated genes.

Table 2. Three groups of WKY rats subjected to Affymetrix GeneChip analysis

Group Name	Steroid treatment	ANFH
Group 1 (G1)	**–**	**–**
Group 2 (G2)	**+**	**+**
Group 3 (G3)	**+**	**–**

Table 3. Differentially expressed genes from comparing group 2 (G2) versus 1 (G1).

Accession #	Gene name	PV	FC	R
NM_012488	alpha-2-macroglobulin	0.0005	3.52	+
NM_012929	collagen, type II, alpha 1	0.0005	2.52	+
NM_030852	melanoma inhibitory activity 1	0.0008	2.29	+
NM_033499	scrapie responsive gene 1	0.0054	2.08	+
NM_017094	growth hormone receptor	0.0142	1.93	+
NM_053669	SH2B adaptor protein 2	0.0213	1.89	+
NM_080698	fibromodulin	0.0099	1.87	+
NM_133523	matrix metallopeptidase 3	0.0034	1.87	+
NM_012999	Proprotein convertase subtilisin/kexin type 6	0.0117	1.80	+
NM_138889	cadherin 13	0.0049	1.77	+
NM_031808	calpain 6	0.0086	1.73	+
NM_001002826	murinoglobulin 2	0.0022	1.72	+
NM_145776	solute carrier family 38, member 3	0.0040	1.71	+
NM_012846	fibroblast growth factor 1	0.0441	1.70	+
NM_017058	vitamin D receptor	0.0065	1.69	+
NM_001009662	carbonic anhydrase 8	0.0275	1.68	+
NM_031590	WNT1 inducible signaling pathway protein 2	0.0105	1.67	+
NM_012587	integrin binding sialoprotein	0.0276	1.66	+
NM_053816	calcitonin receptor	0.0316	1.63	+
NM_013191	S100 protein, beta polypeptide, neural	0.0123	1.62	+
NM_031828	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	0.0018	1.62	+
NM_133569	angiopoietin-like 2	0	1.62	+
NM_199398	pannexin 3	0.0032	1.62	+
NM_053605	sphingomyelin phosphodiesterase 3, neutral	0.0126	1.62	+
NM_170668	solute carrier family 13 (sodium-dependent citrate transporter), member 5	0.0167	1.60	+
NM_053977	cadherin 17	0.0233	1.60	+

NM_199407	unc-5 homolog C (C. elegans)	0.0002	1.60	+
NM_012620	serine (or cysteine) peptidase inhibitor, clade E, member 1 (also designated plasminogen activator inhibitor-1 or PAI-1)	0.0003	1.60	+
NM_022667	solute carrier organic anion transporter family, member 2a1	0.0055	1.59	+
NM_001034009	melanoma cell adhesion molecule	0.0032	1.58	+
NM_053288	orosomucoid 1	0.0236	1.57	+
NM_031131	transforming growth factor, beta 2	0.0015	1.57	+
NM_013059	alkaline phosphatase, liver/bone/kidney	0.0218	1.57	+
NM_133303	basic helix-loop-helix domain containing, class B3	0.0114	1.56	+
NM_198768	immunoglobulin superfamily, member 10	0.0467	1.55	+
NM_001017479	transmembrane protein 100	0.0431	1.54	+
NM_020073	parathyroid hormone receptor 1	0.0370	1.54	+
NM_024400	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	0.0086	1.54	+
NM_001014043	sphingomyelin synthase 2	0.0131	1.53	+
NM_023970	transient receptor potential cation channel, subfamily V, member 4	0.0219	1.52	+
NM_020656	parvin, alpha	0.0072	1.52	+
NM_175578	regulator of calcineurin 2	0.0390	1.52	+
NM_031655	latexin	0.0080	1.52	+
NM_001013218	receptor accessory protein 6	0.0045	1.52	+
NM_001005562	cAMP responsive element binding protein 3-like 1	0.0376	1.50	+
NM_001017496	chemokine (C-X-C motif) ligand 13	0.0140	0.55	_
ENSRNOT00000060250	similar to T-cell receptor alpha chain precursor V and C regions (TRA29)	0.0154	0.64	_
NM_203410	interferon, alpha-inducible protein 27-like	0.0325	0.64	_
NM_001008836	RT1-CE13 // RT1 class I, CE13	0.0157	0.64	_
NM_001002280	MAS-related GPR, member X2	0.0021	0.66	_
NM_001008855	RT1 class Ib gene, H2-TL-like, grc region (N3)	0.0350	0.67	_

PV= p value, FC= Fold change, R= Regulation

Table 4. Differentially expressed genes from comparing group 3 (G3) versus 2 (G2).

Accession #	Gene name	PV	FC	R
NM_001012357	chemokine (C-C motif) ligand 9	0.0371	1.86	+
NM_013153	hyaluronan synthase 2	0.0103	1.70	+
NM_030852	melanoma inhibitory activity 1	0.0082	1.62	+
NM_001012072	protein phosphatase 1, regulatory (inhibitor) subunit 3C	0.0411	1.58	+
NM_001009639	tubulin polymerization-promoting protein family member 3	0.0243	1.56	+
NM_012497	aldolase C	0.0155	1.54	+

PV= p value, FC= Fold change, R= Regulation

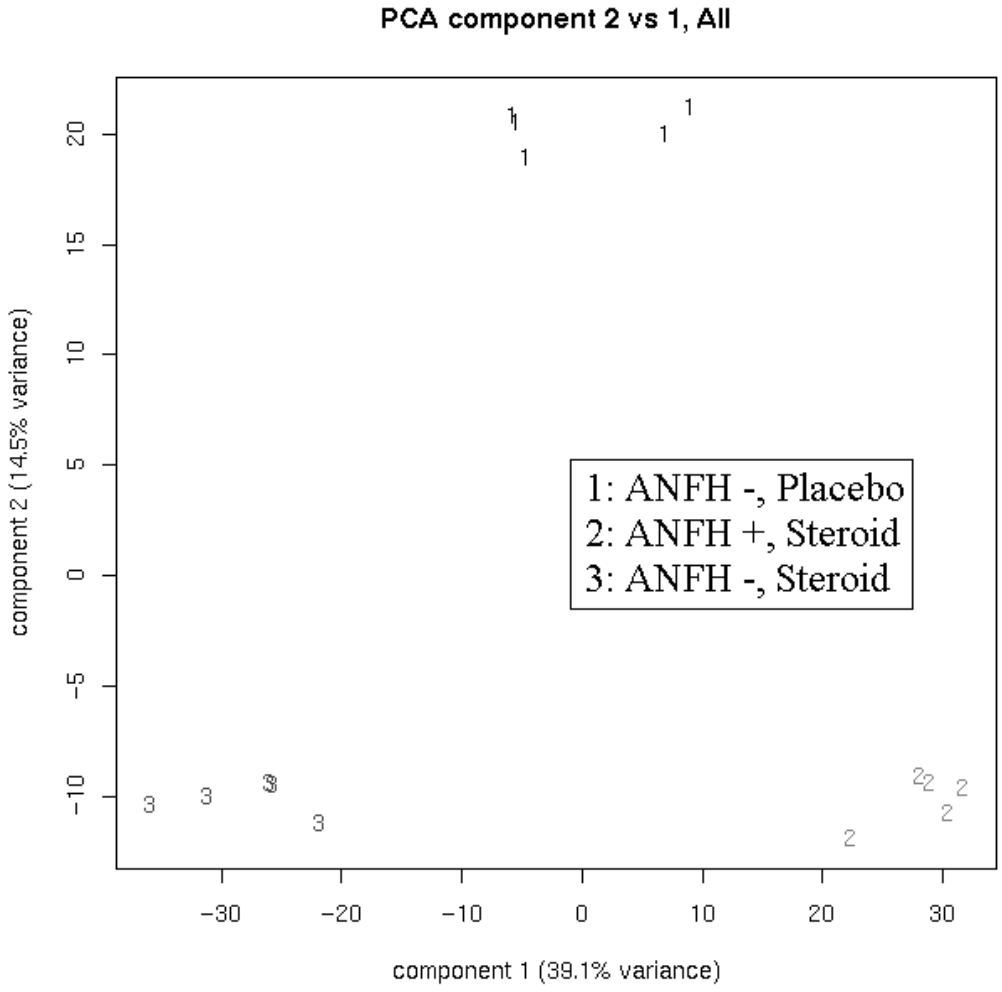

Figure 3. Principle component analysis (PCA) scatter plot showing distinct gene profiling for Group 1 (G1), Group 2(G2) and Group 3(G3). Affymetrix exon array was performed on 15 RNA samples of steroid-treated and non-treated rats divided in 3 groups based on histological evaluation: Group 1- Placebo/ANFH(-); Group 2- Steroid-treated/ANFH(+) and Group 3- Steroid-treated/ANFH(-), each group consisting of 5 samples. Principle Component Analysis of the transcriptional profiles of these 15 samples (G1, G2 and G3), are visualized on a two dimensional plot. The graph depicts component one against component two, which they account for 39.1% and 14.5% of the total variance, respectively. There were no significant differences among the replicants in each group, although there were significant variance among these three groups confirming distinct gene profiling of the 3 groups.

Real time PCR Verification of GeneChip Data

From our microarray results, we then selected the 3 genes that were showing the highest upregulation or fold change when comparing G2 and G1, i.e. α-2-macroglobulin, collagen type II alpha 1, melanoma inhibitory activity 1(Fig. 4), for validation by means of real time PCR. The directional fold change was confirmed for all 3 genes and the correlation with microarray results was established (Table 5).

Likewise, the 3 genes that were showing the highest upregulation or fold change when comparing G3 versus G2, i.e. chemokine (C-C motif) ligand 9, hyaluronan synthase 2 and melanoma inhibitory activity 1, were tested for verification with real time PCR. The results correlated with the microarray data in regards to the directional change, although the differences observed in real time PCR were not stastistically significant (p value: 0.5042, 0.5978, 0.1683, and fold change: 1.24, 1.11, 1.40, respectively). Some variations, however, were noted in the fold-change values demonstrated by real time PCR compared with values obtained by GeneChip analysis. Variations in fold change values between GeneChip and real time PCR might have been due to different methods of normalization and specificity/sensitivity of each method but the trends were the same for the 2 methods.

Immunohistochemistry

We performed immunohistochemistry staining on the 3 candidate genes identified by the Affymetrix GeneChip analysis and RT-PCR method which showed the highest upregulation mainly α-2-macroglobulin, collagen type II alpha 1 and melanoma inhibitory activity when comparing G2 to G1. Protein expression of α-2-macroglobulin was shown to be increased in rats induced with steroids and developing osteonecrosis (Group 2) as compared to the placebo rats without osteonecrosis (Group 1) thus correlating with mRNA expression levels from GeneChip analysis and RT-PCR method (Fig. 5 and 6). However immunohistochemical findings for the 2 other genes of interest (COL2A1 and MIA) failed to show enhanced protein expression (Fig. 5). Considering that MIA was also upregulated when G3 is compared to G2, and that its mRNA expression was more or less equivalent to chemokine (C-C motif) ligand 9 and hyaluronan synthase 2 gene expression (Table 4), FIHC for the latter 2 genes was not performed.

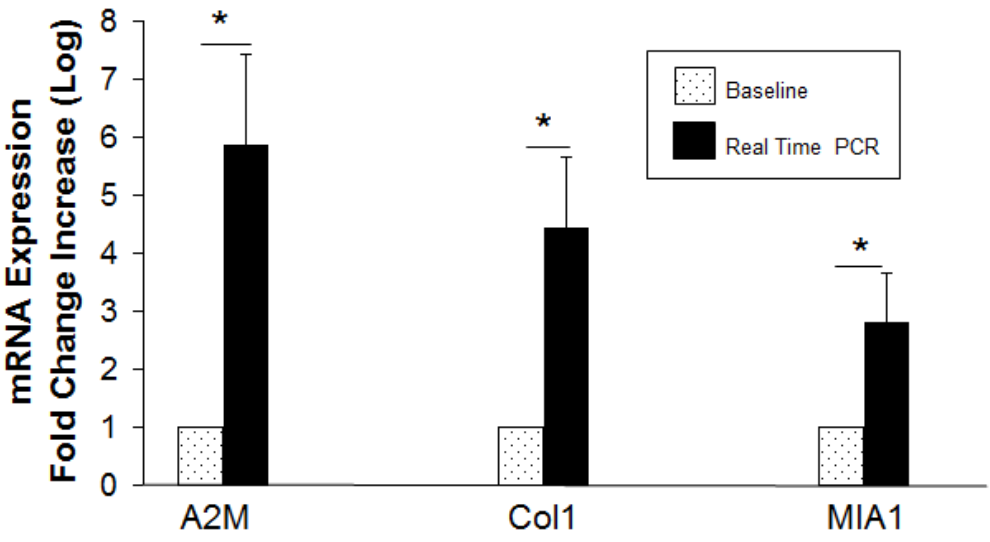

Figure 4. Upregulation of α-2-macroglobulin (A2M), collagen type II alpha 1 (COL2A1), and melanoma inhibitory activity 1 (MIA) mRNA levels in steroid-induced early ANFH. Real-Time PCR assays of A2M, COL2A1, and MIA mRNA expression in steroid-treated rats' femoral head affected by ANFH compared to controls. The selected 3 genes of interest (α-2-macroglobulin, collagen type II alpha 1, melanoma inhibitory activity 1) were significantly overexpressed at the mRNA level. For the relative quantification of gene expression, the comparative threshold cycle (ΔCt) method was employed and normalized against β-Actin rRNA, which was measured by the same method. All PCR reactions were performed in triplicate. *p value: 0.005-0.0009.

Table 5. Correlation of gene expression comparing group 2 (G2) and 1 (G1) as assessed by microarray and Real time PCR ($p<0.05$ for all genes).

Accession #	**Gene name**	**Fold Change of a signal**	
		Microarray	Real time PCR
NM_012488	alpha-2-macroglobulin	3.52	5.85
NM_012929	collagen, type II, alpha 1	2.52	4.42
NM_030852	melanoma inhibitory activity 1	2.29	2.80

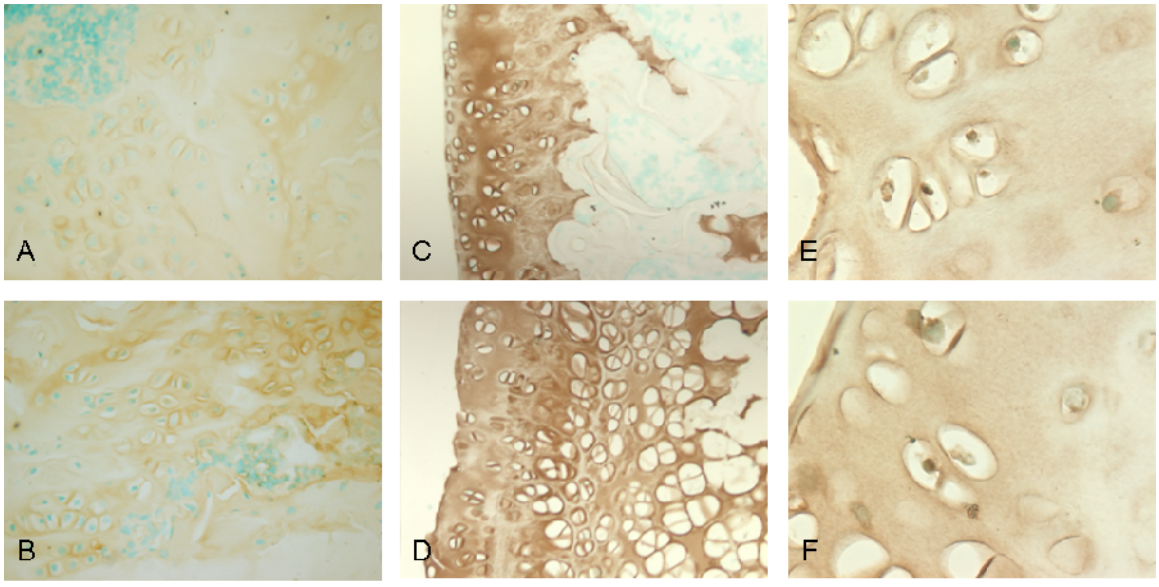

Figure 5. Upregulation of A2M surface protein expression in steroid-induced early ANFH. Fluorescent immunohistochemistry (FIHC) comparing the A2M (**A**,**B**), COL2A1(**C**,**D**) and MIA (CD RAP) (**E**,**F**) protein expression between G1 (**A**, **C**, **E**) and G2 (**B**, **D**, **F**) WKY rats, showing enhancement of A2M expression in G2 compared to G1 but no enhancement shown for COL2A1 and MIA genes; brown color demonstrate protein expression and green color display intact nucleus of cells, **A**-**D** x40 **E**, **F** x100.

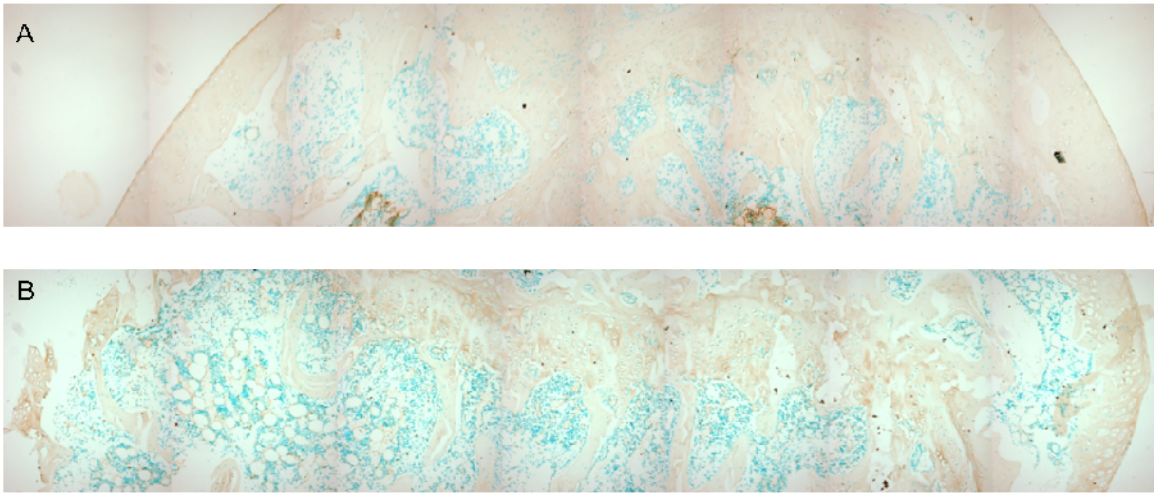

Figure 6. **Upregulation of A2M surface protein expression in steroid-induced early ANFH.** Fluorescent immunohistochemistry (FIHC) comparing the A2M protein expression between G1 (**A**) and G2 (**B**) WKY rats and showing enhancement of expression in G2 compared to G1; brown color demonstrate A2M protein expression and green color display intact nucleus of cells, x100.

Discussion

The early events in the pathogenesis of ANFH are incompletely understood due to the usual late clinical diagnosis. Actualy, radiographic collapse usually occurs before diagnosis. Besides bone marrow changes, evidence has shown that apoptosis is involved in the early stages of steroid-induced osteonecrosis [26]. Weinstein et al. reported that the number of apoptotic cells increased significantly in mice osteoblasts and osteocytes after steroid administration [27]. Recent studies have shown apoptotic cells in clinical and animal models of steroid-induced ANFH [26,28,29]. Therefore, besides bone marrow necrosis which is a common sign in ANFH and reported in the early stages [6], we include the presence of apoptosis in our study as a criterion for diagnosing early disease.

In our previous studies, we characterized an inbred rat strain (Wistar Kyoto, WKY) susceptible to develop steroid-induced osteonecrosis [30]. In the literature, there is a reported 5-15% incidence of spontaneous osteonecrosis in the epiphyseal nuclei of the femoral head in WKY rats at 15 weeks of age [31]. It is possible that this strain of rats has genetic predisposing factors to develop ON and additional risk exposures will facilitate the development of the disease. For instance, repetitive mechanical stress on the femoral heads increases the incidence of the disease up to 33 - 40% [31,32]. In our animal model, prednisone administration enhanced the incidence of the disease in up to 75% (6/8) of the male WKY rats, suggesting it is suitable model to study ANFH. In the literature, 5 -15 week-old rats have been used to study non-traumatic ANFH [23,26,32]. In our study, the WKY rats started to receive steroids at the aged of 5 weeks for a duration of 25 weeks. It has been shown that small rodents such as rats and mice maintain their growth plate into old age [33]. Although this provides the potential for continued longitudinal growth, actually bone growth ceases after a certain time. In rats, the rate of growth increases between 1 and 5 weeks, then reduces until skeletal maturity, that occurs at 11.5–13 weeks [34,35]. Bones still continue to grow, although at lower rate, until around 26 weeks of age, after which growth almost ceases in rats [36]. Therefore, despite the presence of a growth plate in the aged rats, the longitudinal growth no longer occurs. Basically, the morphology of the growth plate changes in aged rats with the existence of sporadic proliferation of chondrocytes [37]. We found that, following a 6-month treatment, the age of 30 weeks is a

suitable age to develop early steroid-induced osteonecrosis. Therefore, for the gene expression study, we purposely selected the rats that presented the same morphology and developing early stage ANFH.

In Affymetrix GeneChip findings, comparison of G2 versus G1 indicated that multiple genes are differentially expressed in presence of ANFH. According to the functional annotation tool (DAVID), modulated genes in the comparison of G2 and G1 (Table 3) were grouped mainly into skeletal development, ossification and bone remodeling. Functional clusters of genes were significantly represented by steroid stimulus response, apoptosis, blood vessel morphogenesis, vasculature development, coagulation-related, cell growth, proliferation and differentiation associated genes.

The expression of steroid stimulus response genes (alpha-2-macroglobulin, alkaline phosphatase, tissue-nonspecific, transforming growth factor beta 2 and potassium large conductance calcium-activated channel, subfamily m, alpha member 1) were expectedly altered significantly. It has been shown in the literature in both *in vivo,* and *in vitro* models and also in clinical studies that steroids induce apoptosis in osteoblasts and osteocytes [29,38-40]. As such, amongst the 51 differentially regulated genes identified in our gene array analysis (Table 3), 5 genes [S100 protein-beta polypeptide, transforming growth factor-beta 2, vitamin D receptor, unc-5 homolog c (c. elegans) and growth hormone receptor] are in fact components of the apoptosis pathway.

Alternately, the process of apoptosis can be directly induced by steroids but could also be resulting from an ischemic condition induced by thrombosis in the blood vessels of the femoral head. Among several confounding pathogenic mechanisms for ANFH, *vascular hypotheses* appear to be the most persuasive, presuming that a decrease in the local blood flow in the femoral head due to vascular obstruction by any means plays a pivotal role in the pathogenesis of ANFH. Damage or activation of endothelial cells, the inner lining of the vascular wall, may result in abnormal blood coagulation and thrombi formation [41]. In keeping with the theory of endothelial cell activation having a role in ANFH, coagulation-related gene expression in particular serine (or cysteine) peptidase inhibitor, clade E, member 1, also called plasminogen activator inhibitor 1 (PAI-1), a serine protease inhibitor that is synthesized and released by endothelial cells in the blood, was shown to be significantly overexpressed in this study. Increase in PAI-1 suppresses

the generation of plasmin resulting in hypofibrinolysis and a relative hypercoagulable state [6]. Decreased fibrinolytic activity, which may be a consequence of increased PAI-1, has been described in patients with ON [42], although a few studies have reported that there were no significant differences in the levels of thrombotic and fibrinolytic factors [19,20].

Similarly, our findings demonstrate that several genes involved in the dynamic remodeling structure of the femoral head are also shown to be differentially expressed in ANFH. Genes modulating cartilage degradation [disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1] and bone loss (parathyroid hormone receptor 1) were upregulated. Alternatively, ossification genes (calcitonin receptor, immunoglobulin superfamily, member 10 and integrin binding sialoprotein), proteoglycan and collagen genes (matrix metallopeptidase 3, fibromodulin, fibroblast growth factor 1, integrin binding sialoprotein, procollagen, type II, alpha 1) were also overexpressed. We hypothesized that if the balance between degradation and repair (bone remodeling) becomes shifted to degradation and bone loss, a failure of structural integrity at the subchondral region could occur. By any means, if the repair improved or degradation was prevented, ANFH could be healed or delayed. It has been shown that in the edematous area of the ANFH adjacent to the necrotic area, VEGF is highly expressed [43], indicating that its expression is correlated with bone tissue repair [44]. Yang et al. used vascular endothelial growth factor (VEGF) gene transfection to enhance the repair of ANFH in a rabbit model of ANFH disease. They observed that bone repair was augmented in the femoral head treated with VEGF gene [45,45].

In the present study, we call attention to novel results showing that alpha-2-macroglobulin (A2M) gene expression was the most significantly upregulated gene when comparing G2 to G1. Correlation was obtained at the microarray, RT-PCR as well as the protein level as demonstrated by FIHC study results. A2M is a plasma-derived matrix metalloproteinase inhibitor which obstructs cartilage degradation induced by matrix metalloproteinases [46]. Thus, in our rat model, corticosteroids seem to modulate significantly A2M levels. The literature supports the role of corticosteroids in the modulation of A2M. For example, it has been shown that dexamethasone modulates A2M in cultured rat liver fat-storing (Ito) cells [47], while another study reports on the effect of corticosteroids on A2M plasma concentration in rats [48]. In both reports, corticosteroids

were shown to enhance A2M levels. A2M is also reported as being implicated in the inhibition of ADAMTS-7 and ADAMTS-12 involved in cartilage degradation [49], while another study in humans demonstrates the role of A2M as an osteogenic growth peptide (OGP) - binding protein. Activated A2M may thus participate in the removal of OGP from the system [50]. Additional reports suggest inhibition of BMP–1 (bone morphogenic protein-1) by native and altered A2M [51]. A2M has also been identified on the luminal surface of endothelial cells in sections of normal human arteries and veins [52] and also in rat[53] and human[54] hepatocytes and more recently, CD109, a novel branch of the alpha 2-macroglobulin/complement gene family, was fully cloned and found to be expressed on endothelial cells, platelets, T-cells and a wide variety of tumors [55,56]. In our study, total RNA was extracted from the femoral head and thus, A2M appears to be overexpressed locally; experimental studies such as in situ hybridization which was not performed could help in the identification of the specific cell type (endothelial cells, osteocyte, osteoblast or chondrocyte etc) expressing A2M. It is thus plausible that its expression derives from the endothelial cells rather than any other cell type located at the femoral head. A2M has also been implicated in hemostasis as a regulator of thrombin [57] and in the development of thromboembolism in children [58]. Together, these findings suggest that A2M shares haemostatic, cartilaginous and osteogenic properties and may have a potential role in the development of early steroid-induced ANFH. Considering that apoptosis is a major step in the cascade of events leading to ANFH, it is interesting to speculate based on our findings, that A2M overexpression induced by steroids could possibly be regulated via one major apoptosis pathway potentially the same way dexamethasone is known to induce apoptosis in proliferative chondrocytes through activation of caspases and suppression of the Akt-phosphatidylinositol 3'-kinase (PI3K/AKT) signaling pathway [59]. Whether A2M overexpression in our study is the result or the cause of the apoptosis found in our rats, developing early ANFH following administration of steroids, requires further study.

Two other genes of interest, collagen type II, alpha-1 (Col2A1) and melanoma inhibitory activity (MIA) respectively, were also shown to be overexpressed significantly by microarray analysis and RT-PCR results but fluorescence immunohistochemical study failed to show an increased cell surface expression of these two genes. Cartilage is made

up of type II collagen which plays a critical role in the development and growth of most bones [60]. Interestingly, Liu YF et al. have reported a G-->A transition in exon 50 (p.G1170S) of COL2A1 in affected members of a Taiwanese four-generation family with ANFH [61]. Also, the missense mutation (p.G1170S) at collagen type II, was also reported in a Japanese family with an autosomal dominant hip disorder suffering ON[11]. On the other hand, melanoma inhibitory activity (MIA), also called cartilage-derived retinoic acid-sensitive protein (CD-RAP), is regulated similarly to collagen type II. MIA (CD-RAP) influences the action of bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)- beta during mesenchymal stem cell differentiation, supporting the chondrogenic phenotype while inhibiting osteogenic differentiation [45]. It has been shown that BMP-2 gene expression decreased after GC treatment and that individuals who were more susceptible to a GC-induced decrease in BMP-2 and osteocalcin gene expression were more likely to develop ANFH [62]. This BMP-2 decrease could be explained by native and altered A2M expression [51].

Comparing the gene profiling of G3 versus G2, 6 genes stood out in our analyses (Table 4). The expression of MIA (CD RAP) was upregulated significantly in the ANFH non-developing group. MIA is hypothesized to have a role in inhibiting osteogenic differentiation [63]. Hyaluronan synthase 2 was also found to be upregulated in the same group. Its synthesis is related to inhibition of osteoblast development [64,65]. Cytokine 9 (CCL9), which acts through chemokine (C-C) motif receptor 1 (CCR1), was also significantly overexpressed. CCL9 and its receptor CCR1 as the major chemokine and receptor species are expressed by osteoclasts, and CCL9 has a crucial role for in the regulation of bone resorption [66]. Although G3 animals have not developed ANFH, their gene profile reflects inhibition of osteoblast proliferation, differentiation and osteoclast activation. This could be explained by the fact that the osteogenic cells in this group have not gone through the apoptotic phase and there are more viable cells expressing these molecules in comparison to G2. Our principle component analysis showing distinct gene profiling findings in treated inbred genetically susceptible Wistar Kyoto rats confronting those developing and not developing the disease, could also be explained by the following. First, the gene expression analysis findings are supportive of a result effect indicating that not only the steroid treatment but also a disease effect likely implicating

the apoptotic process, are involved in the early stages of ANFH. Second, a genetic variation based on single nucleotide polymorphisms (SNPs) could provide an explanation for the phenotypic differences found in our study. Third, an epigenetic variation resulting from the interaction between the genotype and the environment is also a potential process likely to occur which could explain the findings that not all treated animals developed early ANFH when submitted to the same experimental conditions. Fourth, one could argue that any of the genes listed in the comparison of G3 to G2 (Table 4) to the exception of MIA, could have a protective effect against the development of steroid-induced early AVN. Similarly, the absence of A2M overexpression in that same group comparison G3 to G2 (Table 4) is coherent with the phenotypic absence of early ANFH in rats composing G3.

In summary, it is postulated that multiple pathological reactions occurs during ANFH. Genetic predisposition contributes to the development of ANFH as the literature clearly shows that male Wistar Kyoto rats are more susceptible compared to other strains of rats. Of outmost importance in the understanding of ANFH pathophysiology, there is normally a balance between degenerative and regenerative molecules in the bone environment of the femoral head. GCs could trigger a degenerative process as well as inhibiting the repair. In our study, we found that several molecules are significantly upregulated and likely to be involved in the pathogenesis of ANFH. However, only A2M gene overexpression has been consistently found at the microarray, RT-PCR and protein level out of the 3 genes showing the highest upregulation, suggesting that it may play an important role in the development of the early phase of ANFH induced by steroids. Identifying its implications in specific pathways – apoptosis versus endothelial cell dysfunction or both, will likely lead to a better understanding of the molecular events which follow the administration of steroids and subsequent irreversible necrosis and bone collapse. Identifying the underlying pathways by which these genes, and more particularly A2M, exert their effect following steroid administration could help for the development of a preventive strategy of ANFH in patients treated with GCs.

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Chapter 7

Final Discussion

Although the pathophysiology of non-traumatic avascular necrosis of the femoral head (ANFH) pathology remains unclear, most scholars agree that ANFH is associated with thrombus formation in the microvasculature of the femoral head, as depicted in the vascular hypothesis for ANFH (chapter 1). It appears that damage to the endothelial cell membrane, combined in some cases with an increased propensity to blood clot formation, is the key cause of blood flow interruption in ANFH. Under normal conditions, there exists a dynamic balance between fibrinolysis and coagulation in order to maintain the normal blood fluidity and keep blood vessels clear of obstruction. When a thrombus develops, it is typically accompanied by activation of the fibrinolytic system. Plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA) are the two main enzymes involved in the regulation of the fibrinolytic system. Both are primarily secreted by endothelial cells. Alterations in this pathway lead to a destabilization of the fibrinolytic system, with consequent lost of the balance between blood coagulation and fibrinolysis (37). Tan *et al*., using comparative serum proteomic, showed that the levels of expression of t-PA, bone-carboxyglutamate protein (BGP), c-sis, as well as a protein of unknown function were downregulated in the sera of patients with ANFH, whereas the three other proteins, including PAI-1, crosslaps, and anti-p53 antibody, were up regulated. In our Gene Chip array study we also demonstrated that PAI-1 was significantly overexpressed in ANFH animal models.

In a similar proteomic study, Wu *et al*. showed that ANFH patients expressed significantly higher levels of kininogen 1 variant, complement factor C3 precursor, and complement factor H, but lower levels of antithrombin III chain B, apolipoprotein A--IV precursor, and gelsolin isoform alpha precursor. These proteins were reported to modulate

thrombotic/fibrinolytic reactions, oxidative stress, vessel injury, tissue necrosis or cell apoptosis in several tissue types under pathological contexts (40). Although our GeneChip study identified different molecules as being regulated in ANFH, the previously identified pathways were involved. We propose that, in addition to a dynamic balance between fibrinolysis and coagulation, a balance in bone remodeling must be maintained in the micro-environment of the femoral head. Our results indicated that genes modulating cartilage degradation [disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1] and bone loss (parathyroid hormone receptor 1) were upregulated. In addition, ossification genes (calcitonin receptor, immunoglobulin superfamily, member 10 and integrin binding sialoprotein), proteoglycan and collagen genes (matrix metallopeptidase 3, fibromodulin, fibroblast growth factor 1, integrin binding sialoprotein, procollagen, type II, alpha 1) were also overexpressed. We hypothesized that if the balance between degradation and repair (bone remodeling) became shifted to degradation and bone loss, a failure of structural integrity at the subchondral region could occur. This suggests that if the repair mechanisms could be improved or degradation prevented, ANFH could be reversed or delayed. This hypothesis is consistent with studies revealing a correlation between ANFH and expression of vascular endothelial growth factor (VEGF), a major inducer of angiogenesis also important for bone formation (34) and bone repair (32). VEGF regulates bone remodeling by attracting endothelial cells and osteoclasts and by stimulating osteoblast differentiation (12). Yang *et al*. used VEGF gene transfection to enhance the repair of ANFH in a rabbit model of ANFH disease (41,42). It has been shown that in the edematous area of the ANFH adjacent to the necrotic area, VEGF is highly expressed (7). Kim et al. recently reported promoter polymorphisms of the vascular endothelial growth factor (VEGF) gene correlating with the occurrence of ANFH (20).

It is noteworthy that in our study, alpha-2-macroglobulin (A2M) gene expression was the most significantly upregulated gene. In our rat model, glucocorticoids (GC) seem to modulate significantly A2M levels. Previously, a glucocorticoid response element (GRE), a conserved consensus sequence for a putative GC receptor DNA binding site, was found in the 5’-flanking region proximal to the A2M gene promoter (30). The literature also supports the role of glucocorticoids in the modulation of A2M has discussed in chapter 6.

A2M has also been identified on the luminal surface of endothelial cells in sections of normal human arteries and veins (4), and more recently, CD109, a novel branch of the alpha 2-macroglobulin/complement gene family, was fully cloned and found to be expressed on endothelial cells, platelets, T-cells and a wide variety of tumors (23,36). A2M has also been implicated in hemostasis as a regulator of thrombin (11) and in the development of thromboembolism in children (5). We suggest that besides the known cartilaginous and osteogenic properties of A2M, alterations of its hemostatic and coagulative effects are involved in the development of steroid-induced ANFH.

Another family of growth factors highly expressed in bone and involved in chondrocyte and osteoblast differentiation is the TGF-beta superfamily, which consists of TGF-beta, bone morphogenetic proteins (BMPs), activins, inhibins, and other molecules (12). TGF-beta 2 and melanoma inhibitory activity (MIA), also called cartilage-derived retinoic acid-sensitive protein (CD-RAP), were also significantly overexpressed in our study. MIA (CD-RAP) influences the action of bone morphogenetic protein-2 (BMP-2) and transforming growth factor (TGF)- beta during mesenchymal stem cell differentiation, supporting the chondrogenic phenotype while inhibiting osteogenic differentiation (42). It has been shown that bone morphogenic protein 2 (BMP2) gene expression decreased after GC treatment and that individuals who were more susceptible to a GC-induced decrease in BMP2 and osteocalcin gene expression were more likely to develop ANFH (8). This BMP2 decrease could be explained by native and altered A2M expression (44).

On the other hand, collagen type II (COL2A1), another gene found to be overexpressed with ANFH, is regulated similarly to melanoma inhibitory activity (MIA). At the cartilage level, collagen type II forms an arcade of tough fibers that entrap proteoglycans. The proteoglycans absorb large amounts of water to distend the arcade, and the distended arcade gives cartilage its flexibility to compression (33). As mentionned in chapter 6, a G->A transition in exon 50 (p.G1170S) of COL2A1 in affected members of a four-generation Taiwanese family with familial form of ANFH was reported (24). Also, the same missense mutation, has been reported in a Japanese family with an autosomal dominant hip disorder suffering from ANFH (28). The missence mutation (p.G1170S) is considered a predisposing factor to develop ANFH and this genetic variation in the East Asian families may account for the high incidence of ANFH in this region. It has been

proposed that this amino acid substitution could cause conformational changes in the structure and function of the collagen protein, which would influence the molecular interactions of collagen with other extracellular matrix constituents, such as proteoglycans.

The gene expression analysis findings in our study identified several molecules, predominantly A2M, COL2A1 and MIA, which are likely important in the pathogenesis of ANFH. The gene expression analysis findings are supportive of a result effect indicating that not only the steroid treatment but also a disease effect likely implicating the apoptotic process, are involved in the early stages of ANFH.

In summary, we characterized an inbred rat strain (Wistar Kyoto) genetically susceptible to develop steroid-induced osteonecrosis. For the first time, we were able to isolate endothelial cells from the human femoral head bone tissue, thus confirming the presence of endothelium within the cortical bone environment. As glucocorticoids are considered a major cause of osteonecrosis, we were interested in examining better the effect of glucocorticoids on endothelial cell gene expression. Our study showed that GCs, especially at high dose, sensitize human primary endothelial cells to TNF-α- and LPS-mediated neutrophil adhesion and modulate haemostatic gene expression favoring proadhesiveness. These *in vitro* results were in agreement with the current conception of the effects of GCs at the disease level. We then tested the hypothesis that GCs contribute to a differential gene expression in the femoral head of rats with early ANFH, when comparing steroid-treated Wistar Kyoto rats that have developed ANFH versus placebo-treated rats. From the results of this study, it is postulated that multiple pathological reactions occurs during ANFH, and that there is a balance between degenerative and regenerative molecules in the bone microenvironment of the femoral head. GCs appear to trigger a degenerative process as well as inhibiting the repair. Our study also demonstrates that several molecules including MIA, Col2a1 and even more importantly A2M, are significantly upregulated and thus involved in the pathogenesis of ANFH, most likely at the level of the apoptosis pathway. Identifying the specific underlying pathways by which these genes exert their effect following steroid administration will likely contribute to the development of a preventive strategy of ANFH in patients treated with GCs. Further studies may include the identification of a mutation or a polymorphism in

the sequencing analysis of one of the candidate genes especially A2M, and performing genotype-phenotype studies of the knock-out animal model of A2M gene. The later study could prove whether or not A2M plays a definite pathogenic role in ANFH.

Chapter 8

More publications:

We included our other publications in this chapter.

Apoptotic cell death in steroid-induced bone disease: A pilot study in rats

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(Published in: Bone, 2007, Volume 40, Issue 6, Pages: S287-S287)

The underlying pathophysiology of the death of the bone cells in steroid-induced bone diseases such as osteonecrosis is uncertain. The cause of steroid-induced osteonecrosis is thought to be blood flow impairment, but true mechanisms have not yet been elucidated. We have investigated if apoptosis, i.e., programmed cell death, could be an underlying potential mechanism for osteonecrosis of the femoral head in five inbred strains of steroid-induced rats.

We observed that apoptosis occurs at the level of osteocytes, osteoblasts, bone marrow cells and chondrocytes in the early stages of steroid-induced osteonecrosis-like lesions (ONLL) in three strains (Wistar Kyoto, Wistar Furth and spontaneously hypertensive) of inbred rats. Apoptosis could be derived from a direct effect of steroids on the cells or secondary to the dysfunction/stimulation of other cells such as endothelial cells on the bone cells. It is proposed that apoptosis of bone cells is a key pathogenic mechanism in osteonecrosis of the femoral head. The two other strains (Lewis and Fisher) were fairly or

absolutely resistant to develop any ONLL in response to steroid induction, respectively. Our preliminary study in rats suggests that a genetic component is involved in osteonecrosis in response to steroids. Understanding these genetic factors will facilitate the identification of high-risk individuals for osteonecrosis.

Consequently, a primary diagnosis could be made before the disease occurs or progresses further. The apoptotic action of steroids on bone forming cells can also be considered as a potential target for therapeutic intervention of steroid-induced bone diseases.

Vascular etiology of bone diseases: Avascular necrosis

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(Published in: Bone, 2007, Volume 40, Issue 6, Pages: S277-S277)

Avascular necrosis (AVN) of the femoral head in particular is a costly and common disease. Several hundred thousand hip arthroplasties are performed every year for end stage AVN. Surgery is probably not the best treatment for this disease entity. Traditionally, the etiology of AVN has had several explanations. These have included but are not limited to clotting abnormalities, bone formation abnormalities and biomechanical factors at the level of the bone and the attendant skeletal architecture. AVN is manifested terminally by death of bone cells that results in impairment of normal reparative processes along microfractures in the femoral head. This can lead to eventual collapse of the architectural bony structure of the femoral head leading to joint pain and loss of function.

Currently, non-traumatic causes are mainly manifested in idiopathic conditions such as administration of medication or alcohol use. There seemed to be no common pathway to explain the multitude of other causes for AVN (Caisson's disease, sickle cell, graft versus host disease and others). A common pathway for thrombotic abnormality, decreased bone formation and genetic susceptibility may exist in the endothelial cell monolayer of the hip. This constitutes the inner lining of the vascular wall and plays an essential role in the homeostasis of the blood vessel. The concept of a focal nature of a systemic

hematological defect resulting in local hypercoagulable states has been reported. Deficiencies of antithrombin III, protein C and protein S result in deep venous thrombosis of the extremities. TTP and HUS result in microthrombotic lesions that are detectable in all organs except the liver and lungs. Antiphospholipid-antibody syndrome results in clotting of only particular venous and arterial areas including the retina and the placenta. The heterogeneity of the endothelial cell structure amongst these many different organs has been postulated to be a probable cause for the varied clotting responses in the separate endothelial bed. Regional endothelial beds have a different local expression of systemic defects. This regional endothelium dysfunction may be a reason for AVN.

Procoagulant Effect of Dexamethasone in Human Umbilical Vein Endothelial Cells: a Potential Mechanism of Glucocorticoid-induced Osteonecrosis

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Introduction: Glucocorticoids (GCs) are widely prescribed drugs in a variety of diseases. Their use are strongly influenced by their negative side effects. Bone-related effects are mainly osteoporosis and osteonecrosis (ON). Despite the strong link between GCs and ON, the pathogenic mechanisms by which GCs cause ON are still unclear. Cumulative evidence shows that dysfunction or activation of endothelial cells (ECs) play an important role in ON.

Methods: In this study, we investigated the influence of dexamethasone (Dex) on the Tumor Necrosis Factor-alpha [TNF-alpha] or Lipopolysaccharide [LPS] or Thrombin [IIa] -stimulated Human Umbilical Vein Endothelial Cells (HUVEC). We examined the molecular expression of 9 candidate genes (E-selectin [E-Sel], Intracellular adhesion molecule-1 [ICAM-1], Plasminogen activator inhibitor-1 [PAI-1], Tissue Factor [TF], Tissue plasminogen activator [t-PA], Urokinase plasminogen activator [u-PA], Vascular adhesion molecule-1 [VCAM-1], Von Willebrand Factor [vWF] and Thrombomodulin [THBD]) by real-time PCR. Live cell number of HUVEC under exposure to Dex was also assayed by viability test. Experiments were performed in triplicates and Standard error of the mean (SEM) was obtained.

Results: We showed that Dex alone significantly induced the expression of E-Sel, ICAM-1, TF, VCAM-1 and VWF while downregulating THBD and U-PA expression. Our results also showed a significant priming effect of Dex on ICAM-1 (TNF-alpha) and TF (TNF-alpha and LPS) inflammatory-mediated induction and of Dex on THBD (TNF-alpha) inflammatory-mediated downregulation. Northern Blots showed comparable results. FACS analysis and Functional assay results will be presented at the meeting.

Conclusions: Our observations suggest a procoagulant activity of Dex on HUVEC. We also observed a priming activity of Dex on ICAM-1, TF and THBD inflammatory-mediated gene expression. These results suggest a potential endothelial cell activation mechanism and subsequent microvascular thrombosis in glucocorticoid-induced ON.

Isolation and Characterization of Human Bone-Derived Endothelial Cells (hBDECs) for the Study of Vascular Bone Disease States

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Objective: Endothelial cells play an active role in the physiopathology of vascular disease, including thrombus formation. Macro-vessel disease is a well recognized clinical entity with easy tools for measurement of disease extent and treatment of the sequellae. However, micro-vessel disease is not as well understood or diagnosed. Vascular insufficiency at the micro-vessel level and its effect on bone is seen in several disease entities. Chief among these is osteonecrosis. In osteonecrosis of the femoral head, both decreased and then increased vascularity in the subchondral bone has been observed. The initial condition is thought to be decreased vascular supply from a number of secondary factors. This decreased flow is most often the result of interruption of blood supply to the femoral head at the micro-vessels, presumably due to localized femoral head thrombus formation. The effect of the local endothelial cells on this process is currently being defined. Initial research efforts have shown that the bone endothelium may be locally participating in the formation of thrombus, as a result of altered endothelial cell gene expression.

Methods and results: Isolation of endothelial cells from bone was previously reported only in a mice model. There has been no report of the isolation and expansion of endothelial cells from human bone-derived tissue. This paper illustrates a successful isolation and characterization of human bone-derived endothelial cells, using mainly flow cytometry markers, immunochemistry and tubule formation functional assays. These results suggest that it is possible to isolate and identify bone-derived endothelial cells as demonstrated by the positivity for PECAM-1, vWF, VE-cadherin and the formation of tubular structures in matrigel assays – all recognized as endothelial cell specific markers and/or function.

Conclusion: These findings are significant as this strategy may represent the first human bone-derived endothelial cell-based approach to the study of bone diseases such as osteonecrosis.

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Appendix: compliance forms

Claims to Originality

- For the first time, we were able to isolate endothelial cells from the human femoral head bone tissue.
- GCs at high dose modulate human primary endothelial cells expression favoring proadhesiveness and procoagulation.
- Wistar Kyoto and Wistar Furth inbred rats are genetically susceptible to develop steroid-induced osteonecrosis.
- Multiple pathological reactions occur during ANFH.
- Genetic predisposition contributes to the development of ANFH e.g. Wistar Kyoto versus Fisher rats.
- GCs contribute to a differential gene expression in the femoral head of rats with early ANFH.
- Several molecules such as A2M, COL2A1 and MIA1 are significantly upregulated in the femoral head of rats with early steroid-induced ANFH and are likely to be involved in its pathogenesis.
- Since A2M has haemostatic, cartilaginous and osteogenic properties, it may have a potential role in the development of early steroid-induced ANFH.

List of Figures and Tables

Publications

Peer –Review Publications:

1. **Kerachian MA.**, Cournoyer D., Harvey EJ., Chow TY., Neagoe PE., Sirois MG,. Séguin C. (2009*) Effect of High Dose Dexamethasone on Endothelial Haemostatic Gene Expression and Neutrophil Adhesion.* **J Steroid Biochem Mol Biol**. Sep;116 (3-5):127-33.

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