Host-Tumor Interactions in Skeletal Metastasis of Prostate Carcinoma

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Abstract

Approximately 70% of patients with prostatic cancer develop bone metastases. Metastatic prostate adenocarcinomas are associated with high mortality rates and represent a leading cause of cancer-related deaths among males. To study the host-tumor interactions underlying the predilection of prostate cancer cells for skeletal bone, an experimental model was developed using rat Dunning carcinoma Mat-LyLu cells. Inoculations of these cells into the left ventricle of the heart led to the developement of spinal metastases in 100% of inoculated animals. A subline of Mat-LyLu (Mat-LyLu-B5) was subsequently selected through sequential inoculation of bone marrow derived carcinoma cells into the left ventricle of the heart and was found to have an increased metastatic potential compared to the parental line. The possible role of tumor cell adhesion to host cells in the process of bone marrow colonization was then investigated **in vitro** using the metastatic line and primary cultures of rat bone marrow-derived stromal cells. It was found that the adhesion of the metastatic Mat-LyLu cells to a bone marrow stromal cell culture highly enriched for endothelial cells (BMEC) was significantly higher than the adhesion to other bone-derived cells including non-endothelial BM stromal cells (3x) and osteoblasts (1.4x). It was also significantly higher than the adhesion to rat fibroblasts (5.5x) and to hepatic endothelial cells (7x). The results suggest that the adhesion of prostate carcinoma cells to the bone marrow endothelium may play a role in their metastasis to bone.

Résumé

Les patients porteur d'un cancer de la prostate développent des métastases osseuses dans 70% des cas Les adénocarcinomes de la prostate métastasique sont associés à des taux élevés de mortalité et représentent la principale cause de décés dus au cancer chez les mâles Pour l'étude des interactions hôte-tumeur en tenant compte de la prédeliction des cellules du cancer de la prostate pour l'os squelettique, un modèle éxpérimental a été développé en utilisant des cellules du carcinome du rat Dunning Mat-LyLu L'inoculation de ces cellules dans le ventricule cardiac gaucne entraine le développement de la métastase de la colonne vertebrale chez 100% des animaux inoculés Une sous lignée des cellules Mat-LyLu (Mat-LyLu-B5) a été sélectionnée suivant des innoculation répétées, dans le ventricule gauche cardiac, de cellules cancéreuses, dérivant de la moêle osseuse Elle a été demontrée possédant un potentiel métastasique plus important que sa lignée parentale Le rôle possible de l'adhesion de la cellule tuméreuse aux cellules hôtes dans le processus de la colonnisation de la moêle osseuse a été exploré *in vitro* en utilisant une lignée métastasique ainsi que de cultures primaires de cellules stromales dérivées de la moêle osseuse Il a été démontré que l'adhesion des cellules métastasiques Mat-Lylu aux cellules stromales de la moêle osseuse, mise en culture enrichie pour les cellules endothéliales (BMEC), était significativement plus élevée que l'adhesion à d'autres cellules dérivées de l'os y compris les cellules BM stromales non-endothéliales (3x) et les ostéoblastes (1.4x). L'adhesion est également plus élevée que celle aux fibroblastes du rat (5.5x) et aux cellules endotheliales hépatiques (7x) Ces résultats laissent suggérer que l'adhesion des cellules cancéreuses de la prostate à l'endothelium de la moêle osseuse doit jouer un rôle dans leur métastase à l'os.

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Contributions to the Original Knowledge

1. A rat model of prostate carcinoma metastasis to bone was developed using the Dunning carcinoma cell line and intra-cardiac injections.

2. The prostate carcinoma cells were significantly more adherent to Bone Marrow endothelial cells than to primary cultures of fibroblasts, hepatic endothelial cells and osteoblasts.

3. Prostate carcinoma cells selected through 5 consecutive Intra-cardiac injections of bone metastasizing cells were significantly more metastatic and more adherent to the Bone Marrow endothelial cells than the parent line.

4. A significant increase in adhesion to fibronectin and vitronectin was also observed compared to laminin, osteopontin, collagen type I & collagen type IV.

List of Reagents

Tissue Culture Reagents

Collagenase	: Boehringer-Mannheim, Montreal, QC
Dexamethasone	: SIGMA, St-Louis, MO, U.S.A.
Dil-Ac-LDL	: Biomedical Technologies, Stoughton, MA
DMEM	: GIBCO Laboratories, Burlington, ONT
DNAse	: SIGMA, St-Louis, MO, U.S.A.
Endothelial Cell Growth Factor	: Endo-Growth, Vec Tec, New, York, USA
Fetal Bovine Serum	: GIBCO
Fibronectin (Rat)	: Collaborative Research, MA, U.S.A.
Gentamycin	: SIGMA, St-Louis, MO, U.S.A.
Glutamine	: MA Bioproduct
Heparin	: Organon, Toronto, ONT
Laminin	: Collaborative Research, MA, U.S.A.
McCoy's 5A Medium	: GIBCO, N.Y. U.S A.
Osteopontine	: Gift from Dr.Charles Prince, University of Alabama at Birmingham, Birmingham AL, U.S.A.
Penicillin-Streptomycine	: GIBCO
PBS	: GIBCO

PBS-EDTA	: GIBCO
RPMI	: GIBCO
Trypsin	: GIBCO
Type I &	
Type IV Collagen	: Collaborative Research, MA, U.S.A.
Vitronectin	: SIGMA, St-Louis, MO, U.S.A.

Radioisotope

$Na^{51}Cr$	: NEN Research Products, Dupont Canada, Mississauga, ONT

Antibodies

Rabbit Antisera	
to vWF	: Dakopatts, Glostrup, Denmark
Swine Anti-rabbit	
Immunoglobulin	
TRITC-conjugated	: Nordic, Capistrano Beach, CA

List of Abbreviations

Ac-LDL	: Acetylated low density lipoprotein
BAE	: Bovine aortic endothelial cells
BPH	: Benign hyperplasia of prostate
BMSC	: Bone-marrow stromal cells
BMEC	: Bone marrow stromal cells enriched for endothelial cells
CSFs	: Colony Stimulating factors
DES	: Diethyl-stilbestrol
DRE	: Digital rectal examination
Dil-Ac-LDL	: Ac-LDL labeled with the fluorescent probe 1, 1'-dioctadecyl-1-3, 3,3',3',-tetramethyl-indocarbocyanine perchlorate
DMEM	: Dulbecco's modified Eagle medium
EDTA	: Ethylenediamine tetraacetic acid
FACS	: Fluorescent activated cell sorter
FBS	: Fetal bovine serum
Fibro	: Fibroblasts
FITC	: Fluorescein isothiocyanate
HEC	: Hepatic endothelial cells
HGF	: Hematopoetic growth factors.
HUVE	: Human umbilical vein derived endothelial cells
mAb	: Monoclonal antibodies

NSS	: Normal swine serum
Osteo	: Osteocyte
PTH	: Parathyroid Hormone
PBS	: Phosphate buffered saline
PSA	: Prostate specific antigen
RT	: Room temperature
SD	: Standard deviation
TRAP	: Tartate resistant acid phosphatase
TRITIC	. Trimethylrhodamine isothiocyanate
uPA	: Urokinase plasminogen activator
vWF	: Von Willebrand factor

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Preface

The guide lines concerning Thesis Preparation issued by the Faculty of Graduate Studies and Research at McGill university read as follows:

" It is acceptable for theses to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instances, connecting texts are mandatory and supplementary explanatory material is always necessary. Photographs or other materials which do not duplicate well must be included in their original form".

" The thesis should be more than just a collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final over all conclusion".

I have chosen to write my thesis according to the above quoted option with a paper published recently. The thesis is organised in five chapters. Chapter I-III contain general introduction and literature review. Chapter IV contains introduction, materials and methods, results, discussion and references of a manuscript published in Cancer Research. Chapter V contains the summary, conclusions and suggestions for future studies.

Publication Arising from the Work of this Thesis and Contributions Made by Co-authors

Mahmudul Haq, David Goltzman, Gilles Tremblay and Pnina Brodt.

Rat Prostate Adenocarcinoma Cells Disseminate to Bone and Adhere Preferentially to Bone Marrow-Derived Endothelial Cells.

Cancer Research 52: 4613-4619, 1992.

The candidate was responsible for carrying out the experiments described in this manuscript. FACS analysis was done at the Transplantation Biology Lab., The Royal Victoria Hospital and Dr. G. Tremblay, at Department of Pathology helped with the analysis of histological sections.

CHAPTER I

Prostate Cancer : A Review of Clinical Aspects

1.1 Introduction

The alarming rate of increase of prostate cancer each year is a serious medical problem (Carter, H.B., and Coffey, D S , 1990). It has already surpassed lung cancer as the most prevalent malignancy in North American males (Chiarodo, A , 1991) and is the second major cause of cancer related deaths in men (Carter, B S., Carter, H B., and Isaac, J.T., 1990). In the US, prostate cancer requires 250,000 hospitalizations, costs more than $ 1 billion and results in over 28,000 deaths annually (American Cancer Society, 1989, Silverg, E., et al 1990 and Chiarodo, A. 1991) In Canada, the numbers are equally staggering with over 10,000 cases being diagnosed in 1990, the Canadian male has an 8% chance of developing prostate cancer and 3.2% chance of dying from it (Canadian Cancer Statistics, 1990).

1.2 Epidemiological Aspects

The wide range of incidence of prostate cancer among racial or ethnic groups reflects one of the striking features of the epidemiology of this disease US black males mortality, due to prostate cancer, is twice that of white males and they have the highest incidence in the world (Ross, R K , et al , 1988). The reasons for this are unknown, though one study found a 15% higher testosterone level in young adult black men (Ross, R.K., et al., 1986). Nonetheless, these differences cannot be explained only by genetic predisposition, since prostate cancer rates increase substantially in migrants to the US from low risk countries, such as Japan

Perhaps it should be more important to study tumor promoting influences in

determining the malignant potential of prostate cancer rather than the malignant transformation itself.

The death rates from prostate cancer also vary widely around the world. The highest death rates occur in men in northwest Europe, namely in countries such as Sweden, Norway, and Switzerland, while the lowest rates occur in far Eastern countries, such as Hong Kong and Japan (Page and Asire, 1985).

1.3 Risk Factors

One third of all males over the age of 50 years carry prostate cancer in its hidden or latent form. Presently, over 10 million US males harbor this form. This is a unique feature of prostate cancer and it is uncertain how or when this form is activated to the more aggressive form (Franks, L.M., 1954). Autopsy data show that histologically apparent adenocarcinoma is found with increasing frequency from age 40 years onward (Dhom, G , 1983). As the life expectancy of US males increase, so will the risk for prostate cancer (Chiarodo, A., 1991).

1.4 Clinical Presentation

Sufferings from prostate cancer is also of great concern since it is associated with long period of infirmity and bone metastasis which usually produces severe pain that is difficult to control. A long, unpleasant, and expensive medical course is a result of this type of cancer as, 10% of the patients can live up to 10 years, while 80% survive 2-5 years. Many older patients may die of other causes while still burdened with

prostate cancer.

1.5 Metastasis

The high mortality from prostate cancer results from the fact that 80% of patients have metastatic disease, and 30% of them have distant metastases at the time of initial presentation (Scardino, P.T., 1989 and Chiarodo, A , 1991). Moreover, 10% of these patients will die within a year. Prostate cancer cells disseminate through lymphatic and hematogenous routes to the lymph nodes, liver, lungs, and skeletal bone. Up to 70% of prostate cancer patients will develop bone metastasis, the vertebral column being the most commonly affected (Boland, P.J , et al , 1982, Bos, G.D., et al., 1988). Bone metastasis is not only an indicator of bad prognosis, but also a major cause of suffering in the patients causing severe pain, vertebral instability, disruption of neural function with loss of urinary or rectal sphincter control, and, in extreme cases, even complete paraplegia (Harrington, K D , 1988, Bos, G D , et al, 1988).

The factors which underlie the predilection of prostate cancer to bone are unknown, and have become the focus of recent investigation in several laboratories A serious drawback in understanding the biology of prostate metastasis has been the absence of appropiate animal models. This is mainly because skeletal metastasis was rare in animals inoculated with existing prostate carcinoma cell lines. The availability of such models could aid in the study of the biology of this phenomenon and in the development of diagnostic and prognostic methods currently unavailable.

1.6 Diagnosis

1.6.1 Screening and Early Detection

Early diagnosis is the only available method that allows us to treat the disease at a stage for which current therapies are effective. The goal of a screening or early detection program is to detect the clinically important cancers. Several diagnostic tools, including digital rectal examination (DRE), transrectal ultrasound, serum prostate-specific antigen (PSA), serum prostatic acid phosphatase, and flow cytometric analysis are used to detect and monitor this disease (Drago, J.R., 1989). Unfortunately, none of these tests, is adequately sensetive and specific for screening purposes when used alone (Thompson, I.M., and Fair, W.R., 1989). DRE, for example, which is the most widely used screening method for prostate cancer detection offers limited sensitivity because the physician can palpate only the posterior and lateral aspects of the gland, whereas tumors also may occur in portions that are inaccessible to the examiner (American Cancer Society, 1988; McNeal, J.E., 1969), and various benign lesions initially may be misinterpreted as carcinoma (Thompson, I.M , et al., 1984).

1.6.2 Cellular Markers

For understanding and managing prostate cancer, cellular markers offer unique opportunities. Initially, prostatic acid phosphatase, an organ-specific protein, and now PSA have been the best tumor markers (Bilhartz, D.L., et al., 1991; Catalona, W.J.,

et al., 1991). An elevated serum PSA level is present in many prostate cancer patients however it may also be raised in non-malignant conditions such as benign prostatic hyperplasia (BPH) and prostatitis (Stamey, T.A., et al. 1987, Hudson, M A , et al., 1989; Guillet, J., et al., 1988). Men with serum PSA less than 4 µg/L represent the patients with the lowest incidence of prostate cancer (Cooner, W.H , et al , 1990) but a PSA value greater than 50 µg/L probably indicates carcinoma of the prostate and seldom prostatitis (Thomson, R.D., 1992). Moreover a more sensitive PSA assay could help in assessing new therapeutic strategies (Bazinet, M , et al , 1988). However, presently, the PSA has false-negative rate with respect to initial diagnosis and also when used to monitor progression in significant number of cases (Chiarodo, A., 1991).

1.6.3 Imaging

In the past, imaging was not that useful in the diagnosis of prostate cancer But the recent development of many imaging tools such as endorectal ultrasound has helped to identify many prostate cancers (Lee, F , et al., 1989) However endorectal ultrasound, despite marked improvement in resolution, does not adequately differentiate malignant from nonmalignant lesions and its positive predictive value also varies depending upon the clinical findings. Moreover, available ultrasound technology fails to detect many clinically important cancers.

1.6.4 Staging

Accurate staging of prostate cancer is essential in order to determine its malignant potential before administrating any therapy. The treatment approach usually varies, depending on the size of the cancer, local spread, lymph node involvement, and/ or distant metastatic deposits. At present staging is based on clinical determinations which include imaging. But imaging presently can only identify gross, i.e. macroscopic pathology (Chiarodo, A., 1991).

1.6.5 Needle Biopsy

Needle biopsy of the prostate is also one of the important common methods of detecting a suspicious lesion. Needle biopsy under ultrasound guidance is rapidly gaining popularity now-a-days. However only 50% of biopsy specimens collected from prostatic abnormalities reveal adenocarcinoma and significant false negative needle biopsy specimens have also been reported in a number of patients (Bissada, N.k., et al., 1977).

1.7 Treatment

1.7.1 Hormone Therapy

The growth of prostate cancer in adults is thought to be androgen dependent and a tumor often regresses if androgens are withdrawn. However, in most of cases the

tumor recurs even in the absence of androgens. The underlying mechanism(s) by which androgens regulate prostate cells is not yet clear. The current treatment of choice for metastatic prostate cancer is androgen deprivation, such as orchidectomy, estrogen therapy, or luteinizing hormone-releasing hormone (LHRH) agonists and/ or antiandrogens. Treatment usually retards the progression of cancer growth with relief of symptoms (Tolis, G., et al., 1984; Carpentier, P.J., et al., 1984; Spirnack, J.P., et al; 1984; Watanable, H., 1986). But unfortunately, a significant number of patients do relapse as androgen-independent cancer cells which are highly resistant to therapy survive. Although bilateral orchidectomy rapidly removes the major source of circulating androgen such as testosterone, many patients find castration psychologically unacceptable because of subsequent loss of libido and impotence (Rioja, L.A., and Sanz, J., 1991). Administration of therapeutic estrogens reduce the pituitary release of luteinizing hormone (LH), thereby removing the stimulation of testes for the synthesis of testosterone. Although this mode of treatment is simple, the side effects are serious such as gynecomastia and adverse cardiovascular events (Altwein, J.E., 1983).

1.7.2 Chemotherapy

Chemotherapy is not effective against prostate cancer. This might be due to presence of low proliferative fraction of prostate cancer cell population or specific mechanisms of drug resistance yet to be discovered. Therefore, cancer chemotherapy has provided disappointing and debatable success rates so far (Chiarodo, A., 1991).

1.7.3 Radiotherapy

Radiotherapy is an alternative to prostatectomy in the treatment of localized disease. Those in favour of this therapy claim that it is a safe, non-invasive technique and can help patient avoid incontinence. However resistance to radiotherapy is common and complications include chronic symptomatic cystitis, urethral stricture, urinary incontinence and impotence.

1.7.4 Surgical Treatment

Radical prostatectomy is the common mode of surgical therapy but it may also fail due to the presence of undetectable metastatic disease. Therefore, in order to improve the survival rate it requires detection before spread, combined with total radical prostatectomy or complete sterilization of the localized tumor by radiotherapy (Chiarodo, A., 1991).

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CHAPTER II

Skeletal Architecture

and Skeletal Metastases

2.1 Introduction

Bone is a highly vascular, mineralized special connective tissue. It is remarkable for its hardness, characteristic growth mechanisms and its regenerative capacity. All bone tissue consists of cells embedded in an organic matrix with inorganic salts. Bone may be developed either directly from mesenchymal tissue or from cartilagenous tissue replaced by bone later on (Simmons, D.J., 1990).

2.2 Cellular Aspect

Bone cells are responsible for all active processes of bone formation and resorption throughout life. There are two main groups of bone cells; osteoblasts responsible for bone formation, and osteoclasts for resorption. The osteocyte is an inactive osteoblast entombed within the growing bone.

Osteoblastic cells are believed to be derived from the mesenchymal tissue that also gives rise to chondrocytic and myoblastic cells of connective tissue (Grigoriadis, 1988; Stein, et al , 1990). The characteristics of osteoblasts are to produce Type I collagen, alkaline phosphatase, osteocalcin, osteonectin, osteopontin, specific growth factors. Other phenotypic qualities include cAMP-responsiveness to PTH (parathyroid hormone), CT (calcitonin), PGE_2 (prostaglandins E_2) and the stimulation or expression of receptors for PTH and $1,25(OH)_2$ D_3 (Wrana, et al., 1988; McCarthy, 1988, Owen, et al., 1990; Stein, et al., 1990; Friedenstein, 1990; Weinreb, 1990).

Osteoclasts are considered to be derived from the haemopoietic stem cell (Yamashita, T , et al., 1990). They are multinucleated cells with a specialized ruffled

border and a high content of tartate-resistant acid phosphatase (TRAP) expressing calcitonin receptors (Yamashita, T., et al., 1990). Furthermore, they are thought to be derived from fusion of mononuclear precursors (Holtrop, M.E., 1990).

2.3 Composition of the Bone Matrix

The bone matrix is composed of an organic matrix and inorganic salts (Table I. Simmons et al, 1990). The organic matrix is made up of collagenous fibres embedded in ground substances which consist of sialo-proteins and proteoglycans mainly chondrointin sulfate and hyaluronic acid. The inorganic salts incorporated into the matrix are calcium phosphate in crystalline form with magnesium, potassium, sodium carbonate and citrate ion.

While mature bone consists entirely of Type I collagen, osteoblast-like cells derived from neonatal mouse calvariae (MC3T3-El cell. Hata et al , 1984; Kumegawa et al., 1983; Scott et al., 1980) or fetal rat calvaria (Aubin et al., 1982, Bellows et al ., 1986) were also found to produce small quantities of type III collagen in vitro.

2.4 Bone Marrow and Stromal Cells

The bone marrow consists of two histologically distinct compartments: the vascular compartment, comprised chiefly of a system of vascular sinusoids; and, a hemopoietic compartment (Fig. 1), which forms irregular columns between the vessels (Tavassoli, M., and Yoffrey, J.M., 1983). The haemopoietic compartment consists of precursor blood cells which differentiate to give rise to lymphocytes,

Table I. Approximate Composition of Mineralized Bone

Protein Composition of Cellular Fraction	
Soluble Collagen	25%
Blood-derived Proteins	25%
Albumin	
Fibronectin	
Cellular Components	15%
Nucleic acids	
Glycogen	
Cell Protein	
Matrix/Cell Components	
Proteoglycans	10%
Protein core (38 kD)	0.1%
$Alpha_2$ HS-Glycoprotein	
Osteocalcin (57 kD)	
Osteonectin (32 kD)	
Phosphoprotein	
Sialoprotein (70-80 kD)	24%
Unidentified	10%
Glycoprotein (ex. BMP)	

Composition of Mineralized Fraction	
Phosphoglycoprotein (62 kD)	10%
Proteoglycans	7%
$Alpha_2$ HS-Glycoprotein	25%
Osteocalcin (57 kD)	15-20%
Osteonectin (32 kD)	8%
Phosphoprotein (24 kD)	8%
sialoprotein (25 kD)	8%
Unidentified	5%

(Adapted from Simmons, D.J., 1990).

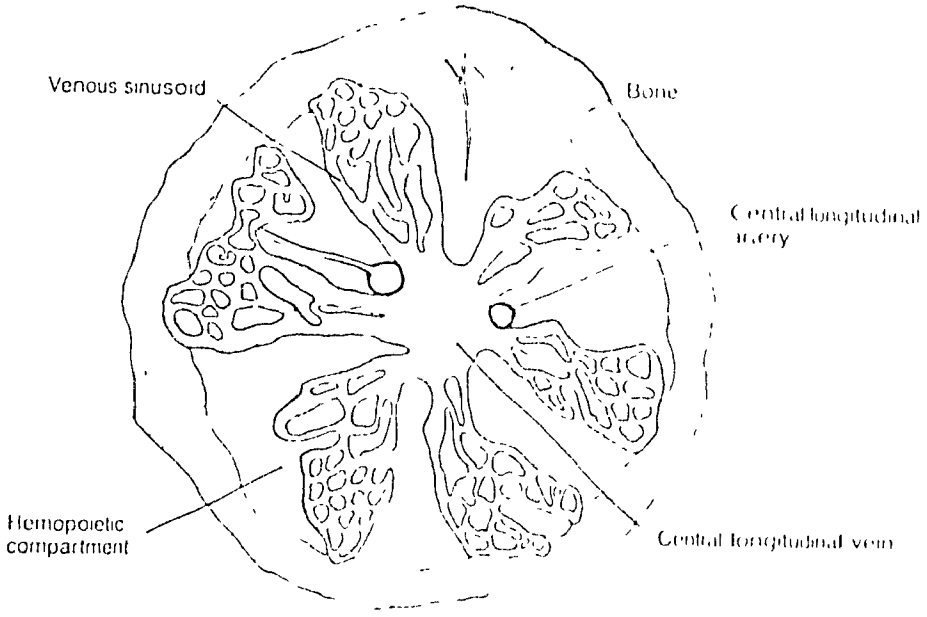

Fig. I. Schematic drawing of a cross section of bone marrow showing its subdivision into a hemopoietic and vascular compartment. (Reproduced from Simmons, D. J., 1990).

erythrocytes, granulocytes, megakaryocytes and monocytes (Owen, 1978; 1980).

The hemopoietic cells are transient in the marrow and move into the blood stream upon maturation. The stromal component which consists of a highly vascular connective tissue, however, remains and helps to differentiate and mature the hematopoietic cells. Studies with long-term in vitro cultures of human bone marrow revealed that the stromal adherent layer was composed of 60%-70% fibroblastic cells, 10%-20% endothelial cells, 10%-20% monocyte/macrophages and 5%-10% fat-laden adherent cells (Strobel et al., 1986). A number of hematopoietic growth factors (HGFs) are known to be produced by the stromal cells. Some of them have already been purified, sequenced and cloned (Kincade et al., 1989).

Although stromal cells establish the architecture of bone marrow and organize hemopoiesis, the interrelationships among the various stromal cell types such as macrophage, fibroblastic, endothelial, and adipocyte-like components are poorly understood. Using monoclonal antibodies it has been observed that the fibroblastic cells derived from rat bone marrow culture differed from those of non-hemopoietic organs and expressed a unique antigen identified by mAb ST3, whereas lung, diapharagm, and epididymal fat pad fibroblasts expressed more ST4 than ST3 (Sullivan et al , 1989).

2.5 Bone Formation and Resorption

The process of bone formation and resorption during bone growth in early life is considered "modelling". This is replaced in adulthood by "remodelling". a continuos process necessary for the maintenance of normal bone structure.

A remodelling site is initiated by the activated osteoclasts following humoral or local stimuli for resorption (Fig. 2). The osteoclasts proceed to resorb an amount of bone producing a small resorption pit (Howship's lacuna, or longitudinal cutting cone in cortical bone). The actively motile osteoclast moves to another site after having resorbed a certain amount of bone. This resorptive phase is followed by an active reversal phase when the cement line is deposited (Baron et al, 1980) During the subsequent formative phase, active cuboidal osteoblasts appear and begin to deposit uncalcified matrix (osteoid) which is later mineralized This process of bone resorption followed by an equal amount of formation has been termed "a basic multicellular unit of bone turnover" (Frost, 1984). It is believed to be regulated by locally produced chemical signals.

2.5.1 Osteoblastic Metastases

The bone is a common site of metastasis for several malignancies, such as carcinomas of the prostate, breast, thyroid, lung and kidney (Mundy and Spiro, 1981). Although skeletal resorption is most commonly observed in association with secondary metastases to the bone, prostatic carcinoma, frequently gives rise to osteoblastic lesion (Franks, L.M., 1973) These lesions are seen in nearly every cases of prostatic bone metastasis suggesting a paracrine regulation (Falasko, C S B., 1976). The finding that the growth of osteoblastic cells is stimulated in vitro by the extracts of prostatic tissue initiated the investigation into the pathogenesis of osteoblastic metastases of prostatic carcinoma (Jacobs, S C., 1979, 1980). Subsequently, mRNA from the prostatic cancer cell line, PC-3, injected into Xenopus

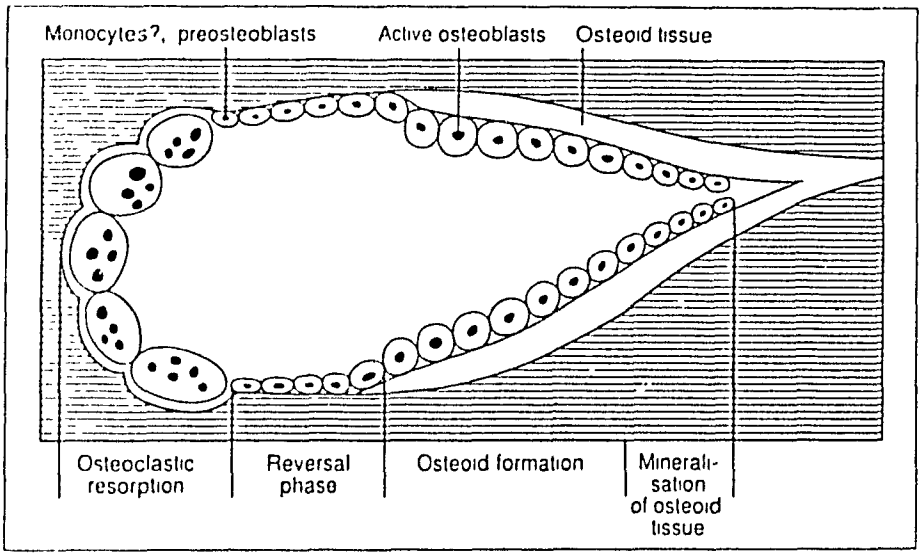

Fig. 2. " Bone remodelling unit " in the Haversion envelope. (Reproduced from Remagen, W., 1989).

oocytes lead to the secretion of a protein which was found to be mitogenic for osteoblastic cells (Simpson, E., 1985). Later studies revealed the presence of growth factors in prostatic tissue which appear to be specific for the osteoblastic cells (Koutsilieries, m., et al., 1986, 1987). Very recently a 15 KDa protein with mitogenic activity for osteoblastic cells has been isolated from the conditioned media of a human prostatic cancer cell line, PC-3, and chemically recognized as an amino-terminal fragment (ATF) of the urokinase plasminogen activator (uPA) (Rabbani, S.A. et al., 1990; Rabbani, S.A., et al., 1992). The involvement of uPA in the production of both osteoblastic and osteolytic skeletal lesions of prostatic cancer may be due to the presence of both growth factor activity and proteolytic activity in its two discrete molecular domains (Goltzman, D., 1992). Thus it is possible that the catabolic activity of uPA helps in breaking down the extracellular skeletal matrix and in causing the osteolytic lesions. In addition, it may work along with other factors, such as parathyroid hormone related peptide (PTHRP), and cytokines which activate osteoclastic bone lesion (Goltzman, D., et al , 1989; Mundy, G N , 1988, and as shown in Fig. 3). The ATF, on the other hand, might be involved in osteoblastic proliferation. The increased frequency of osteoblastic metastases associated with prostatic carcinoma could be due to overproduction of ATF or to neutralization of proteolytic activity by plasminogen activator inhibitors released by the tumor itself (Goltzman, D., 1992).

2.5.2 Osteolytic Metastases

In addition to the potential action of uPA discussed above, tumor cells could

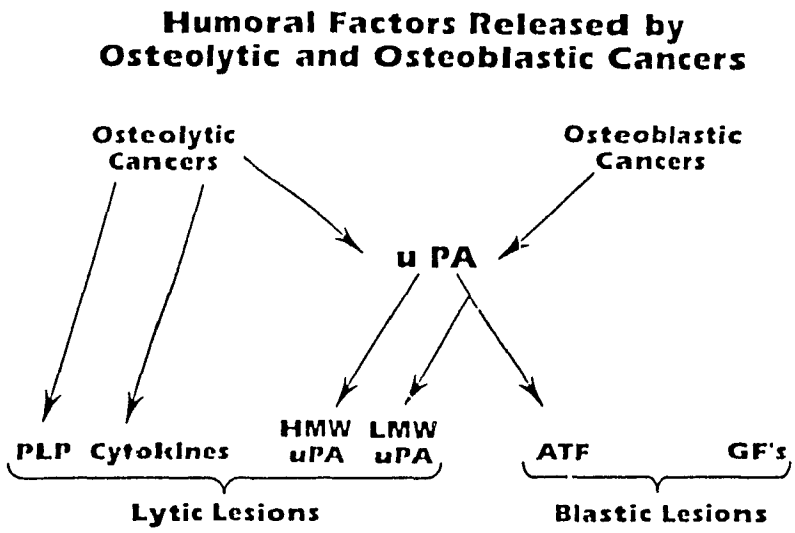

Fig. 3. Model for the potential role of uPA and other peptide factors in the pathogenesis of osteoblastic and osteolytic lesions. (Reproduced from Goltzman, D., et al., 1992). uPA = Urokinase plasminogen activator; HMW = High molecular weight; LMW = Low molecular weight; PLP = Parathyroid hormone related peptide; ATF = Amino terminal fragment; GF = Growth factors.

cause bone resorption by a direct effect on bone. The mechanism is believed to be due to release of lysosomal enzymes and collagenase (Eilon and Mundy, 1979). In general, activated osteoclasts in metastatic disease are larger and have more nuclei (Fig. 4).

In metastatic bone lesions, either more osteoclasts are formed or the survival of the existing ones is prolonged under the influence of continuous activation from tumor cells (Galasko, 1981; Bonucci, 1981). One of the possible mechanisms of bone destruction might be due to the direct or indirect effects of tumor cells on the vasculature leading to necrosis (Cramer, S., et al.,1981) Furthermore, it may result from tumor products (Eilon, G., et al., 1983; McDonald, D F., et al., 1983) or from osteoclastic stimulation by prostaglandin E_2 (Gebhardt, M.C , et al., 1985). Several osteoclast activating factors (Durie, B.G.M., et al., 1981) which probably are a variety of cytokines including interleukins and tumor necrosis factor (Bertolini, D.R , et al., 1986), transforming growth factor α (Ibbotson, K.J., et al., 1985), parathyroid hormone like substances (Rabbani, S.A., et al.,1986; Burtis, W.J., et al.,et al.,1987; Stewart, A.F., et al., 1987; Moseley, J.M., et al., 1987), and perhaps other as-yet unidentified agents, (Canalis, E., 1985) as illustrated in Fig. 4., might be involved in the genesis of osteolytic lesion.

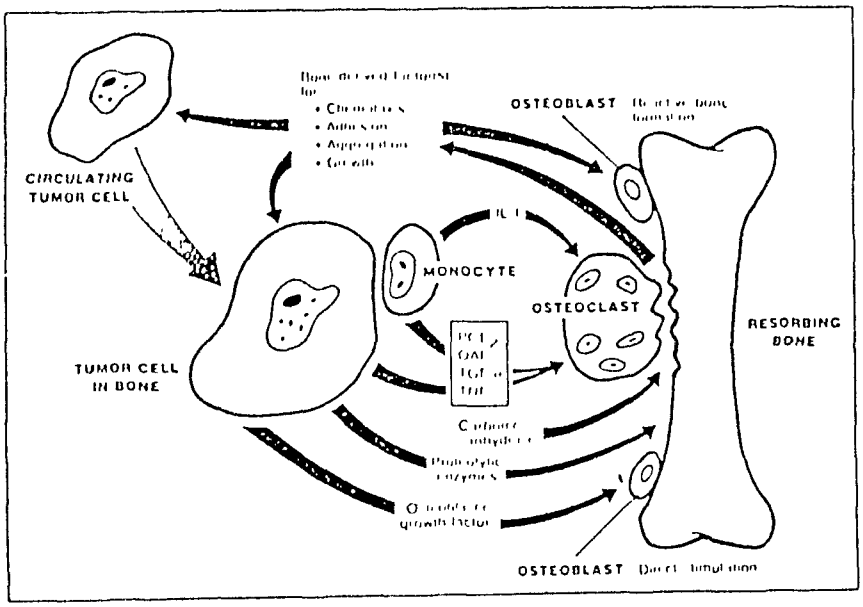

Fig. 4. Schematic drawing of proposed interaction between tumor cells, host mononuclear cells, lymphokines, bone resorption and formation. PGE_2 = Prostaglandin E_2, OAF = osteoclast-activating factor, TGF-α = transforming growth factor alpha, IL-1 = interleukin-1, TNF = tumor necrosis factor. (Reproduced from Scher, H.I., and Yagoda, A., 1987).

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Chapter III

Mechanisms of Metastasis :

A Review of Current Concepts

3.1 Introduction

The ability to metastasize may be considered the most harmful characteristic of malignant tumors. Local control of a primary tumor can be accomplished now-a-days by the use of surgery and radiation but tumors that spread, causing distant metastases, are almost invariably fatal (Posner, 1990).

Bone metastasis is usually selective in terms of the site of origin and of distribution. Eighty percent of cancers that spread to bone are those arising from breast, prostate, thyroid and kidney carcinomas(Cadman, E., and Bertino, J R., 1976) Metastatic foci in all bones are situated mainly in red bone marrow (Willis, R.A., 1973), with the spine being mostly involved. Within the spine, vertebral bodies of the lumbar region are affected most often (Bhalla, S K., 1970; Willis, R A , 1973; Drew, M., Dickson, R.B., 1980; and Enneking, W.F., 1983).

3.2 Organ-Specific Metastasis

It has been frequently observed that some tumors preferentially metastasize to specific organs of the body (Paget, 1889; Zetter, 1990) Different theories have been put forward to explain this phenomenon.

The most popular, "seed and soil" hypothesis of Paget (1889) suggests that metastatic cells, like the seeds of a plant, can only survive or grow if they find a favourable organ ("soil"). In support of this hypothesis, a number of tumor cell lines that have an increased ability to colonize a particular organ have been obtained by in vivo selections (Nicoson, 1983).

"Mechanical theory", proposed by Ewing (1928), suggested that special vascular channels draining the primary tumor determine the ultimate route and site of dissemination of the metastatic cells leading to increase metastases in one organ over another. In support of Ewing's proposal, several investigators have been able to demonstrate that the organ patterns of experimental metastasis is frequently determined by the vascular route of tumor cell injection, as shown with non-lymphohematopoietic tumors in animal models (Murphy et at., 1986; Arguello et al., 1988).

3.3 The Role of the Vertebral Circulation

The vertebral venous channel or Batson's plexus is a special valveless venous communication that runs parallel to the vertebral column and form anastomoses with the venous system of the vertebrae, pelvis, thorax, and brain (Batson, O.V., 1940). Vertebral venous system has been thought to be the main route through which metastatic cells get access to the vertebral column (Lemort, M. et al., 1986; Harrington, K D., 1988). Batson suggested that metastatic cells in circulation might spread to the vertebral column as a result of venous reflex during raised intrathoracic or intraabdominal pressure, such as in coughing, straining, and sneezing (Batson, O.V., 1940). Several investigators have demonstrated that raising the intraabdominal pressure or ligating the inferior venacava while inoculating tumor cells through the tail vein induced vertebral metastasis in animal models (Shevrin, D.H.,et al., 1988; Geldof, A.A., 1990), while injection of the cells from the parent line subcutaneously or via the tail vein without caval occlusion did not result in bone lesions (Shevrin, D.H., et al.,

1986). Although, these experiments can induce bone metastasis to the lumbar spine, wide spread bone metastasis, commonly encountered in the clinical experience, is not reproduced by this method (Kamby, C. et al., 1987). Moreover, venous obstruction is rarely seen in prostate cancer patients. Nevertheless, the metastatic focus in these animal models began from emboli in the anterior vertebral vein of the spinal canal and spread from there to compress the spinal cord or invade the vertebrai body (Arguello, F., et al., 1990). In contrast, as seen in human autopsy studies, tumors compressing the spinal cord mostly arise from vertebral bodies (Fornasier, V.L., 1975). Thus, the importance of venous reflux of cancer cells in the development of vertebral metastases is still debatable in clinical cancer.

3.4 Seed and Soil Hypothesis

Within the vertebral bone hematopoietic bone marrow is the target tissue and primary soil for proliferation of the metastasizing cancer cells. High volume of bone marrow in the lumbar spine might be responsible for the higher incidence of metastasis in that part of the skeleton (Thrall, J.H., and Ellis, B I., 1987). The presence of hematopoietic bone marrow seems to be essential for the formation of bone metastasis in humans (Willies, R.A., 1973). In addition, the rate of perfussion of bone marrow is significantly higher (510 mL/kg per minute) (Johnston, A D , 1970) than that of bone (only 11 mL/kg per min) facilitating the increased accessibility of cancer cells to bone marrow.

The increased frequency of metastatic deposits in the marrow may be explained to some extent on the basis of special structural and hemodynamic factors

associated with bone marrow (Arguello, F., et al., 1988; Berrettoni, B.A., and Carter, J. R., 1986). Moreover, several growth factors produced in the marrow were found to stimulate the growth of many cancer cells in vitro (Berbel, W.E., et al., 1989). Thus, bone marrow appears to be a fertile soil for metastatic cells.

A thorough review of positive bone scintigrams from different cancers including breast and prostate revealed no difference in overall distribution among the various cancers (Dodds et al., 1981) . The authors of this study concluded that systemic circulation might be primarily responsible for the dissemination of malignant cells to different target organs.

Several investigators infact recently demonstrated that inoculations of tumor cells into the systemic circulation of syngeneic or nude mice results in the development of bone metastasis which simulate the human pathology of prostate carcinoma (Arguello, F., et al., 1988, 1990, 1991; Kjonniksen, I., et al., 1990).

3.5 Growth Factors

One of the most important factors which renders a "soil" of an organ congenial to tumor growth is probably the presence of specific cytokines that promote cell division. A variety of growth factors are known to stimulate the growth of normal and neoplastic cells in culture (Deuel, T.F., 1987). Some are more abundant in one organ than in others. For example, there is more of the acidic form of fibroblast growth factor in neural tissue such as the brain, hypothalamus, pituitary and retina. Similarly, transforming growth factor beta is found in platelets, cartilage and bone. Likewise, bone marrow contains numerous hematopoietic growth factors.

Bone metastasis of prostate carcinoma may also be regulated by specific growth factors. This became more apparent when the conditioned media harvested from bone marrow stromal cells was found to be mitogenic to human prostate carcinoma cells (Chackal-Roy, M., et al., 1989). This may also explain the increased growth rate of prostate cancer cells in this secondary site.

3.6 Cell-Cell Adhesion / Interaction with the Parenchymal Cells

The majority of tumor cells are arrested in the first organ, but metastatic foci are often found in other organs. This has led many investigators to speculate that a specific cellular adhesive interaction might be necessary for the initiation of metastases at their target organs.

Evidence now confirms this hypothesis and demonstrates that metastatic tumor cells preferentially adhere to organ-specific adhesion molecules. The initial experiments show that metastatic tumor cells adhere preferentially to disaggregated cells (Nicolson, G L., et al., 1975) or to histologic sections (Netland, P.A , and Zetter, B.R., 1984) prepared from secondary organs. In addition, tumor cells selected for their increased adhesion to lung tissue in vitro were found to have enhanced metastais to lung sites in vivo (Idem, 1985). There is also evidence of adhesion of tumor cells to parenchymal cells of the target organ (Otto, P., et al , 1985, Brodt, P., et al., 1989, 1992) which might be necessary for metastasis to proceed (Zetter, B.R., 1990). These experiments cofirm that cell adhesion is a critical determinant of site-

specific tumor metastasis.

3.6.1 Endothelial Cells

Tumor cells in the circulation first come in contact with the surface of the endothelium and occasionally with exposed basement membrane, where they might adhere to specific adhesion molecules. Several endothelial adhesion molecules have so far been isolated such as ELAM-1, ICAM-1, ICAM-2, VCAM, GMP-140, and PECAM (Bevilacqua, M.P. et al , 1989; Simons, D.L., et al., 1988, 1990; and Staunton, D.E., et al, 1989). Several types of blood cells such as neutrophils, monocytes and lymphocytes in circulation to adhere to the vessel walls through these receptors. Since this represents the first stage by which cells migrate through the vessel wall to the surrounding tissue, these molecules play a crucial role in the process of inflammation and are also thought to be important in tumor metastasis (Staunton, et al., 1989).

Greene and Harvey were the first to propose that organ specific colonization of metastatic cells might depend on the formation of initial bonds between tumor cells and the adhesion molecules on the surface of endothelium lining the vessels of that organ (Greene, H.S.N., and Harvey, E K., 1964). Auerbach and coworkers have subsequently confirmed this theory in a series of experiments (Auerbach, R., et al., 1987). The metastatic cells, in most of the cases, adhered preferentially to the endothelial cells derived from the secondary site. These results suggested that the circulating tumor cells could specifically recognize unique adhesion molecules expressed on the surface of the vascular endothelium of the target organ. It has been

shown that adherent cells are protected from cytotoxic T cell injury and therefore survive longer in the circulation (Kaminski, M., and Auerbach, R., 1988) In addition, cell adhesion might enhance cell motility thereby facilitating invasion of tumor cells into the organ parenchyma (McCarthy, J B., et al., 1985 and Nicolson, G.L., 1991).

3.6.2 The Extracellular Matrix

Tumor cells can also trigger endothelial cell retraction (Zetter, B.R., 1990), and adhere to molecules of the subendothelial matrix (extracellular matrix). Metastatic tumor cells can bind to components of the extracellular matrix (ECM), such as fibronectin, laminin, and Types IV and V collagen (Mc Carthy, J B , et al. 1985; Juliano, R.L., 1987). These ECM proteins may vary from organ to organ Metastatic tumor cells grow preferentially on ECM extracted from the preferred organ site as shown by Doerr and coworkers (1989). Adhesion molecules expressed on the surface of endothelial cells can also be modulated by ECM components derived from specific organs. It has been demonstrated by Pauli and Lee in 1988 that tumor cells that selectively colonize one organ preferentially bind to the aortic endothelial cells grown on ECM extracted from that particular organ Thus, it appears that expression of organ specific adhesion molecules on the vascular endothelium may not be an intrinsic feature of the endothelial cells of that organ, rather regulated by the elements of the ECM on which they grow.

3.7 Rationale and Purpose of Present Study

The role of tumor cell adhesion to the target organ microenvironment in the process of site-specific metastasis has been established by many investigators as mentioned above Although this has been well documented for liver and lung metastasis, little is known about the role of cell-cell or cell-matrix adhesion in prostatic carcinoma metastasis to bone. This is partly due to the lack of appropriate animal models.

The objectives of the present work were therefore as follows:

(1) To develop an animal model for the study of bone metastasis of prostate carcinoma.

(2) To study the role of cell-cell and cell matrix adhesion in tumor-host interactions in bone metastasis.

(3) To identify the cellular components of the host bone and/ or bone-marrow which are involved in the adhesion.

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CHAPTER IV

Rat Prostate Adenocarcinoma Cells Disseminate to Bone and adhere Preferentially to Bone Marrow-Derived Endothelial Cells.

Parts of this chapter were published in Cancer Research 52: 4613-4619, 1992.

4.1 Introduuction

The incidence of, and mortality from prostate cancer are increasing yearly and carcinoma of the prostate is now the second major cause of cancer deaths in males in the USA. One out of 11 men in the USA is expected to be diagnosed with carcinoma of the prostate and of these a third are expected to die from their cancer (Chiarodo, A , 1991). With an increasingly aging population, the mortality rate could increase again by 50% in the next 15 years (Chiarodo, A., 1991).

The skeleton is the major site of metastasis for prostate carcinoma. Approximately 70% of patients with prostate cancer will develop bone metastases (Chiarodo, A., 1991). These metastases, which are situated predominently in red bone-marrow, most commonly in the spine are generally associated with poor prognosis. The development of new and effective therapeutic regimens for the management of prostate carcinoma depends therefore on a better understanding of the mechanisms which underly the predilection of this malignancy to bone.

In recent years, it has become clear that the selective patterns of secondary organ colonization, characteristic of many types of cancers, cannot be entirely explained by anatomical and mechanical trapping theories of tumor cell dissemination (Nicolson, G L , 1988, and Sugarbaker, E.V., 1981). Several specific factors have been shown to regulate this process known as site-specific metastasis (Zetter, B.R., 1990). Among them the preferential adhesion of cancer cells to the organ extracellular matrix proteins, parenchymal cells, and in particular organ-specific receptors expressed on the luminal side of the vascular endothelium have all been shown to play important roles (Brodt. P., 1989; Auerbach, R., et al., 1987; and Pauli, B.U., et

al., 1988). These adhesive interactions are thought to trigger tumor cell invasion and promote cellular proliferation (Zetter, B.R., 1990).

To understand the mechanisms underlying the predilection of prostate cancer to bone, we developed an animal model using Dunning prostate carcinoma cell line R3327-Mat-LyLu. The intra-cardiac inoculation of these cells into the left ventricle of syngeneic rats resulted in the development of bone marrow metastases. Bone-marrow derived carcinoma cells as well as the parental line were then used to analyse the role of cell-cell adhesion in this metastatic process.

4.2 Material Methods

4.2.1 Animals

Inbred male Copenhagen rats weighing 150-200 gr were obtained from Harlan Sprague-Dawley Inc.(Indianapolis, IN). Female Wistar Rats weighing 100-150 gr were obtained from Charles River, (Montreal, Canada).

4.2.2 Tumor Cells

The Dunning R3327-Mat-LyLu cell line (Isaacs, J.T., et al., 1986) was obtained courtesy of Dr. J.T. Isaacs (John Hopkins School of Medicine). It was maintained in vivo in Copenhagen rats by s.c. inoculation of 10^6 cells. The animals usually developed lymph node and lung metastases within 3 wks but bone metastases were not observed (Isaacs, J, T. et al, 1981). The tumors were resected when their

diameter reached approx. 3 cm and the tumor cells enzymatically dispersed in a solution of 0 05% trypsin (Gibco). In vitro the cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine (MA Bioproducts), 10% fetal bovine serum (FBS), 100 U/ml of penicillin-streptomycin sulfate (GIBCO, Burlington, Canada), 250 nM dexamethasone and 0.2% gentamycin (Sigma).

TMT-081, a metastatic rat mammary carcinoma line was obtained from Dr. U. Kim (The Rosewell Park Memorial Institute, Buffalo, New York). The maintenance and the tumorigenic and metastatic properties of these tumor cells were described in detail elsewhere (Brodt, P. et al., 1990).

4.2.3 Bone Metastasis Assay

Lumbar bone metastases of the Dunning tumor were obtained following the injection of Copenhagen male rats with 5 X 10^3 or more Mat-LyLu cells into the left ventricle (i.c. injection) as described elsewhere (Stackpole, C.W., et al., 1985). The animals developed hind leg paralysis within 2-3 wks (depending on inoculum size) due to spinal cord compression by tumor cells extending from the vertebral body, as confirmed by histopathology (see below in Results section). Tumor cells were harvested from the lumbar region and maintained in culture for 2-3 wks at which time some of the cells were used in the adhesion assays. The remaining cells were reinjected i.c. into new rats to obtain the next generation of bone-metastasizing cells. This procedure was repeated 6 times.

4.2.4 Osteoblasts

The isolation of primary osteoblasts from rat fetal calvariae was carried out as previously described by Bernier et al (Bernier, S B , et al., 1990). Cell viability was assessed with trypan blue exclusion dye and the cells were plated in 24 well plates at a density of 1.5 X 10^5 cells/well in RPMI containing 10% FBS and cultured for 72 hrs prior to use in the adhesion assays.

4.2.5 Fibroblasts

Primary cultures of rat fibroblasts were prepared from third trimester fetuses using established procedures (Freshney, R.I., 1987). The cells were cultured in RPMI-FBS at a density of 1.5 x 10^5 cells/well in 24-well plates.

4.2.6 Bone-marrow Stromal Cells

Bone-marrow cells were obtained from the femoral bone of Wistar rats by flushing the marrow cavity with 10 ml RPMI-FBS through a 23-gauge needle The marrow suspension was filtered through a 100 µm mesh sieve, centrifuged at 1200 rpm for 10 minutes, resuspended and plated at a density of 10^6 cells /well in 24-well plates (Nunc) or 4 X 10^6 cells/well in 6-well plates (Nunc) Culture medium was Dulbecco's modified Eagle's medium (DMEM, from GIBCO) supplemented with 20% FBS, antibiotics (as described for RPMI) and 0 1 mg/ml heparin (Orgnanon, Toronto, Ont). To enrich BM endothelial cells, some of the cultures were supplemented with 200 µg/ml endothelial cell growth factor (ECGF-Vec Tec, NY, U.S.A) on the day of plating

and thereafter on alternate days for a total period of 3 weeks.

4.2.7 Endothelial Cells

Cultures of bovine aortic endothelial cell (BAE) were prepared using the procedure described by Gospodarowicz et al.,1976. The cells were maintained as described for BM stromal cells in medium supplemented with ECGF.

Human umbilical vein derived endothelial cells (HUVE), obtained as described elsewhere (Gimbrone, M. A , et al., 1974) were kindly provided by Dr.J.Gordon (Department of Surgery, McGill University). The cells were cultured in McCoy's 5A medium (Flow Laboratories) containing 20% FBS. Rat hepatic endothelial cells were isolated and cultured as described in detail elsewhere (De Leeuw, A. M., et al., 1982).

4.2.8 FACS Analysis

4.2.8.1 Uptake of Ac-LDL

Bone-marrow stromal cells (BMSC) cultured for 2-4 weeks with or without ECGF were labeled by incubation with 10 µg/ml DiI-Ac-LDL (Acetylated Low Density Lipoprotein labeled with 1,1'-dioctadecyl 1-1-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate, from Biomedical Technologies Inc. MA) for 4 hours at 37^0 C. The labeled cells were dispersed with PBS/EDTA, centrifuged and resuspended in RPMI-FBS containing 10 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid).

Fluorescent cells were analysed by FACS (Becton-Dickinson FACSTAR) at an excitation wavelength of 514 nm. A total of 10^4 cells from each sample were analysed. Fibroblasts and BAE cells, the latter previously shown to expresss receptors for Ac-LDL (Voyta, J.C , et al., 1984) were used as negative and positive controls respectively.

4.2.8.2 von-Willebrand Factor (vWF)

BMSC were also analysed for expression of von-Willebrand factor. Three weeks old culture were dispersed with PBS-EDTA, centrifuged and resuspended in culture medium containing rabbit antiserum to vWF (Dakopatts, Glostrup, Denmark) at a dilution of 1:100 (normal rabbit serum was used as control) Incubation was at room temperature (RT) for 60 min The cells were washed three times and incubated with 10% normal swine serum (NSS) in PBS for 30 min to block non-specific binding The second antibody namely, trimethyl-rhodamine-isothiocyanate (TRITC)-conjugated swine-anti-rabbit antiserum (Nordic, Capistrano Beach, CA) was added at a dilution of 1:20 (Both primary and secondary antibodies were diluted in PBS containing 3% NSS) for a 30 min incubation at RT. The unbound antibody was washed with PBS and cells were resuspended in DMEM Fluorescent cells were analysed by FACS. Fibroblasts and HUVE cells, the latter previously reported to express vWF (Wagner, D.D., et al., 1982) were used as negative and positive controls respectively.

4.2.9 Adhesion Assays

4.2.9.1 Adhesion to Cell Cultures

Mat-LyLu cells in log phase were labeled with $Na^{51}Cr$. To monolayers of primary rat cell cultures in 24-well plates, 10^5 labeled tumor cells were added for a 60 min incubation at 37^0 C. Non-adherent cells were removed by repeated washing with PBS and adherent cells lysed with 1N NaOH. Radioactivity associated with the lysate was monitored in a gamma counter.

The number of stromal cells/well at the time of the assay was determined by dispersing and counting the cells from three wells. The average number of cells/well was calculated and used to standarize the results which are expressed as the proportion of prostate carcinoma cells which adhered per 150,000 stromal cells.

4.2.9.2 Adhesion to Extracellular Matrix Proteins

The procedure of the adhesion assay has recently been described elsewhere (Nip, J., et al., 1992). Briefly, the microtitre plates were coated with different extracellular matrix proteins such as osteopontin (Gift from Dr. Charles Prince, University of Alabama, AL, USA), laminin, fibronectin, type I, & type IV collagen (all from Collaborative Research Inc , Bedford, MA) or vitronectin (Sigma Chemical Co., St. Louis, MO) Chromium labeled tumor cells were added to each well, and incubated at 37°C for up to 90 min. The non-adherent cells were removed by aspiration and repeated washing with PBS. Adherent cells were lysed with 1N NaOH and radioactivity in the lysate was measured in a gamma counter.

4.3 Statistics

The Student's t-test and the Mann-Whitney U test were used for the statistical analyses.

4.4 Results

During the course of this study Copenhagen rats were inoculated with Mat-LyLu cells by the i.c., i.v. or s c. routes. The inocula ranged from 5×10^3 to 2×10^5 tumor cells. The results obtained following these injections are shown in table I All animals which received i.c. inoculations developed hind leg paralysis (Fig. 1) followed by death 4-5 days later. No animals inoculated i.v or s c with the tumor cells developed hind leg paralysis and metastases were observed in the lungs and lymph nodes only (table-I). In animals inoculated i.c with 2×10^4 - 5×10^5 cells, paralysis was apparent by days 12-14 In autopsies, a distended bladder due to failure of evacuation was usually found (Fig 2). No macroscopic metastases could be detected in any of the major organs except occasionally in the adrenal glands and kidneys Histological examination confirmed that there were metastatic lesions in the bone marrow of lumbar and lower thoracic vertebrae of these animals. A tumor mass was also found in the spinal canal extending from the bone-marrow and compressing the spinal cord Typical histological findings are shown Fig. 3. As is frequently the case for BM metastases of human prostatic carcinoma both osteoblastic and osteolytic lesions of the tumor were observed (Fig 4) Micrometastases were also observed in the adrenal glands and kidneys but were not detected in the prostate gland, seminal vesicles,

Fig. 1. Photograph of a male Copenhagen rat 2 wks following I.C. injection of Mat-LyLu cells showing the signs of hind leg paralysis.

TABLE I. Selective Metastasis to BM of Mat-LyLu Cells Inoculated I.C. into the Left Ventricle.

Type of cells	No. of cells inoculated	Route of inoculation	# of days prior to Hind leg paralysis[c]	Incidence of Metastasis BM	Lung	LN
Mat-LyLu-P[a]	5×10^4	I.C.	14(13-14)	5/5	0/5	0/5
Mat-LyLu-B[b]		I.C.	12(11-12)			
Mat-LyLu-P	2×10^4	I.C.	13(12-14)	5/5	0/5	0/5
Mat-LyLu-B4		I.C.	12(11-13)			
Mat-LyLu-P	5×10^3	I.C.	19(18-21)[d]	5/5	0/5	0/5
Mat-LyLu-B5		I.C.	12(11-14)			
Mat-LyLu-B6	5×10^3	I.C.	12(12-14)	5/5	0/5	0/5
Mat-LyLu-B5	5×10^3	I.V.	N.S.	0/5	5/5	0/5
Mat-LyLu-B5		S.C.	N.S.	0/5	5/5	5/5
TMT-081	5×10^4	I.C.	N.S	0/6	0/6	0/6

NS: Not Seen

BM: Bone Marrow

LN: Lymph Node

[a]Parental Mat-LyLu Line

[b]Bone Metastasis-derived Mat-LyLu Line.

[c]Median & range.

[d]Statistically significant delay ($P < 0.008$) in onset of hind leg paralysis as compared to Mat-LyLu-B5 or Mat-LyLu-B6 as determined by the Mann Whitney test.

Fig. 2. Dissection findings of the male Copenhagen rat after the development of hind leg paralysis showing distended bladder (arrow).

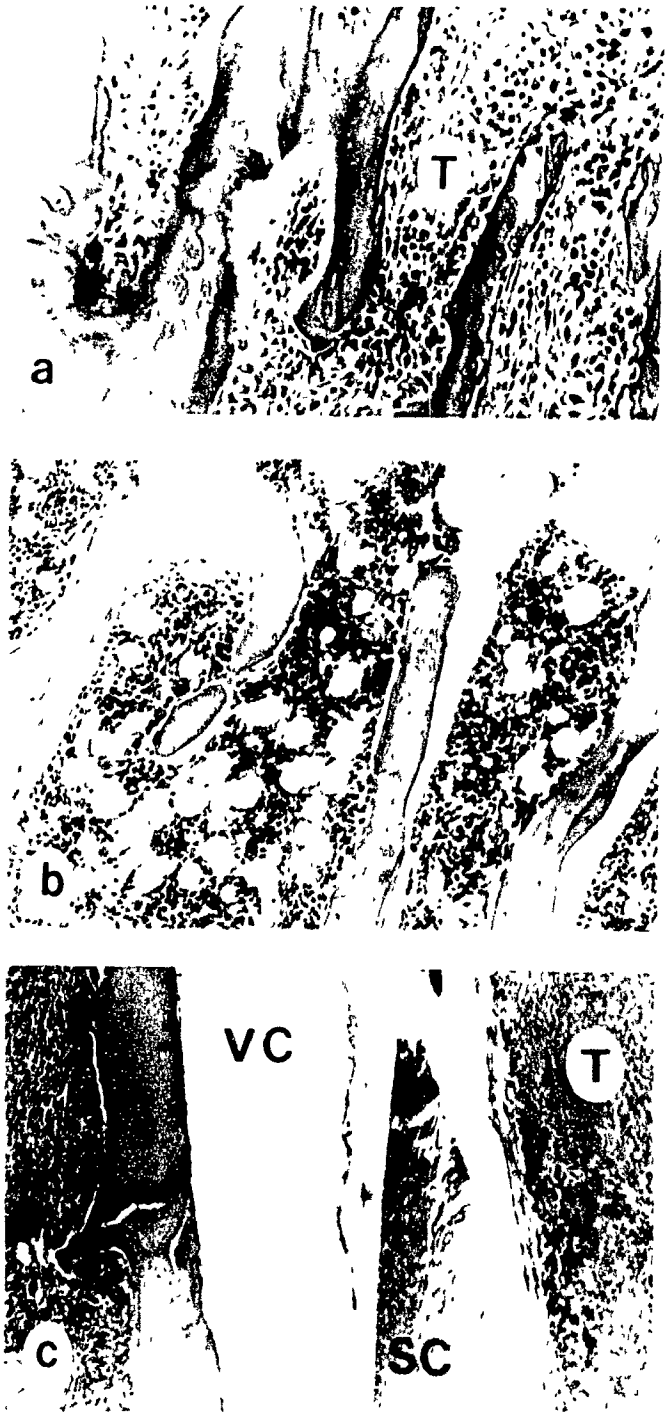

Fig. 3. Light microscopy analysis of a bone metastasis of carcinoma Mat-LyLu Shown in (a) are the histology findings seen in a paraffin section of a lumbar vertebral bone derived from a rat inoculated i.c. with Mat-LyLu cells where bone marrow was totally replaced by tumor (T) A section of a bone derived from a normal animal is seen in (b). In (c) a longitudinal section through the lumbar vertebrae & spinal cord is shown with the tumor mass (T) infiltrating the vertebral canal (VC) and compressing the spinal cord (SC) The vertebrae was removed 2 wks following i.c inoculation of tumor cells when hind leg paralysis was evident. The sections were stained with Hematoxyline/eosin (HE) Magnification: (a) & (b) x 100, (c) x 40

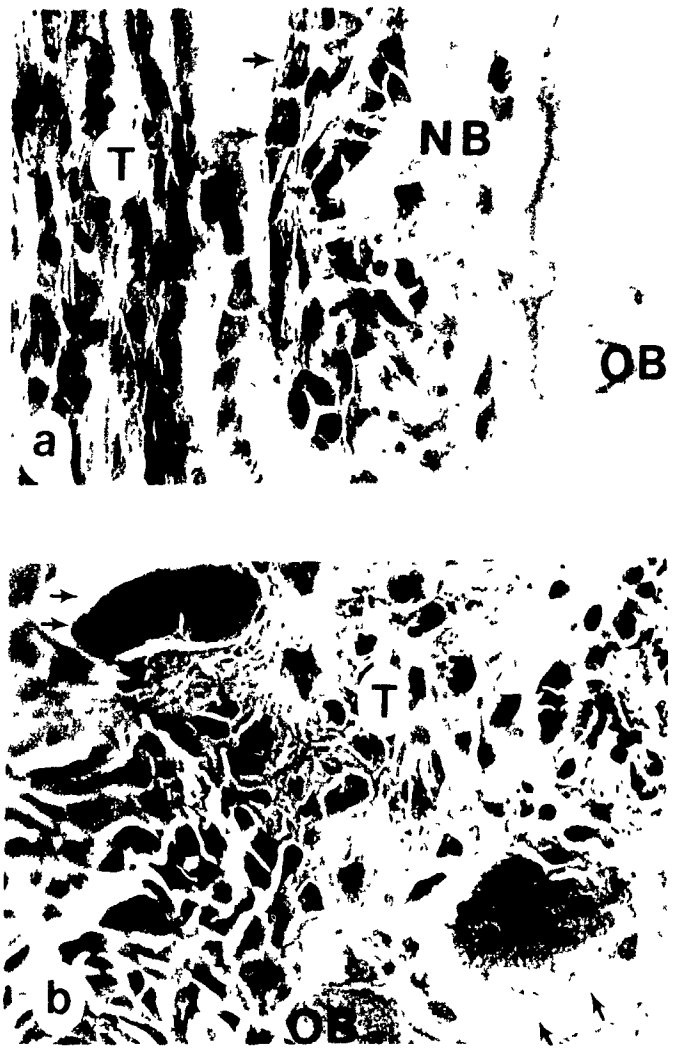

Fig. 4. Characterization of the metastatic lesions of Mat-LyLu in the bone. Shown is a paraffin section of a lumbar vertebrae obtained 2 wks following i.c. inoculation of the tumor cells. In (a) an osteoblastic region is seen with osteoblasts (arrow) and new bone (NB) formation at the interface between tumor mass (T) and old bone (OB). In addition as seen in (b), multinucleated osteoclasts (arrow) with osteolytic lesions were also observed in the tumor mass (T) invading the old bone (OB). H.E. staining. Magnification x 400.

sternum or femurs nor in the lungs, lymph nodes, liver and spleen.

To test whether tumor cells derived through repeated sequential isolation and reinjection of BM-metastases had an increased bone metastasizing potential, the ability of such cells to form BM metastases following i.c inoculation was compared to that of the parental line. As shown in table I, no significant difference in incidence or in the time interval preceding onset of the paralysis was observed when the animals were inoculated i.c. with 2×10^4 - 5×10^5 parental (Mat-LyLu-P) or metastatic (Mat-LyLu-B) cells. However when the tumor cell inoculum was reduced to 5×10^3 cells per animal a significant acceleration in onset of paralysis was noted in animals inoculated with the metastases-derived tumor cells. While rats inoculated with Mat-LyLu-P cells developed paralysis by day 19, rats inoculated with Mat-LyLu-B5 or Mat-LyLu-B6 cells developed paralysis by days 12-14, approximately one week earlier (table I). As a control the metastatic rat mammary carcinoma line TMT-081 was used When 5×10^4 cells of this tumor were injected i.c. into six syngeneic rats they failed to give rise to bone metastases for up to 8 wks following the inoculation (table I).

To study the role of cell-cell adhesion in BM colonization by Mat-LyLu cells, cultures of BMSC were prepared and characterized by cell surface marker analysis Uptake of Ac-LDL has been widely used to distinguish vascular endothelial cells from fibroblasts and mesenchymal cells (Voyta, J.C , et al , 1984) The proportion of Ac-LDL(+) cells in the BM cultures was therefore determined The results shown in Fig 5 and summarized in Table II revealed that there was a significant enrichment of Ac-LDL(+) cells in cultures of BMSC grown in the presence of ECGF

Similarly, when the cells were analyzed for expression of vWF, a significant increase in the proportion of vWF+ cells was noted, as shown in Fig 6 and Table II.

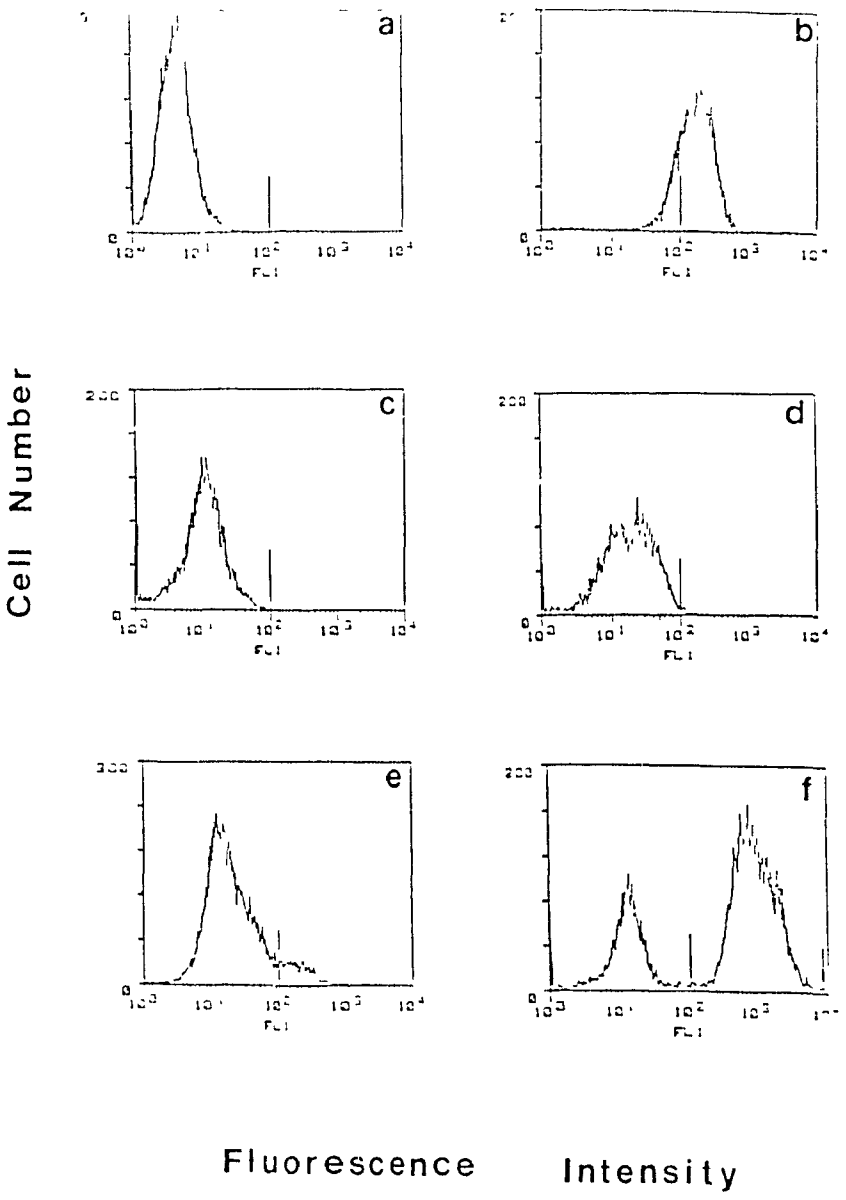

Fig. 5. FACS analysis of rat bone-marrow stromal cells labeled with DiI-Ac-LDL: Cells were labeled with rhodamine conjugated Ac-LDL. The cells on the right of the vertical bar were considered positive. Shown are results of one FACS analysis which were typical of 6 performed (a) fibroblasts. (b) bovine aortic endothelial cells (BAE). (c) bone-marrow stromal cells (BMSC)-unlabeled. (d) bone-marrow stromal cells enriched for endothelial cells (BMEC)-unlabeled. (e) BMSC labeled with DiI-Ac-LDL. (f) BMEC labeled with DiI-Ac-LDL.

TABLE II. Results of FACS Analysis Using Two Different Markers for Endothelial Cell. .

Cells	DiI-Ac-LDL added	MIF[a]	% of +ve cells[b] added	TRITC-anti anti vWF	MIF[a]	% of+ve cells[b]
FIBRO.	+	167	0.1±0.5	+	130	0.4±0.2
BAE/HUVE	+	335	78±4	+	1509	80±3
BMSC	-	74	0.3±0.2	-	117	1.0±0 4
BMEC	-	137	0.5±0.3	-	119	2±1
BMSC	+	215	12±3	+	580	30±6
BMEC	+	1200	70±5[c]	+	1209	65±4.5[c]

FIBRO = Primary culture of fibroblasts.

BAE = Bovine aortic endothelial cells were used as a positive control for Ac-LDL expression.

HUVE = Human umbilical vein endothelial cells were used as a positive control for vWF expression

BMSC = Bone marrow stromal cells cultured without ECGF.

BMEC = Bone marrow stromal cells cultured in presence of ECGF to enrich the endothelial cell subpopulation

[a]MIF = Mean Intensity of Fluorescence Results of experiments described in Fig. 5 & 6 are shown.

[b]Results are expressed as mean and S.D. of 6 experiments with DiI-Ac-LDL and 4 experiments with antibody to vWF.

[c]Statistically significant difference ($P < 0.005$) compared to BMSC.

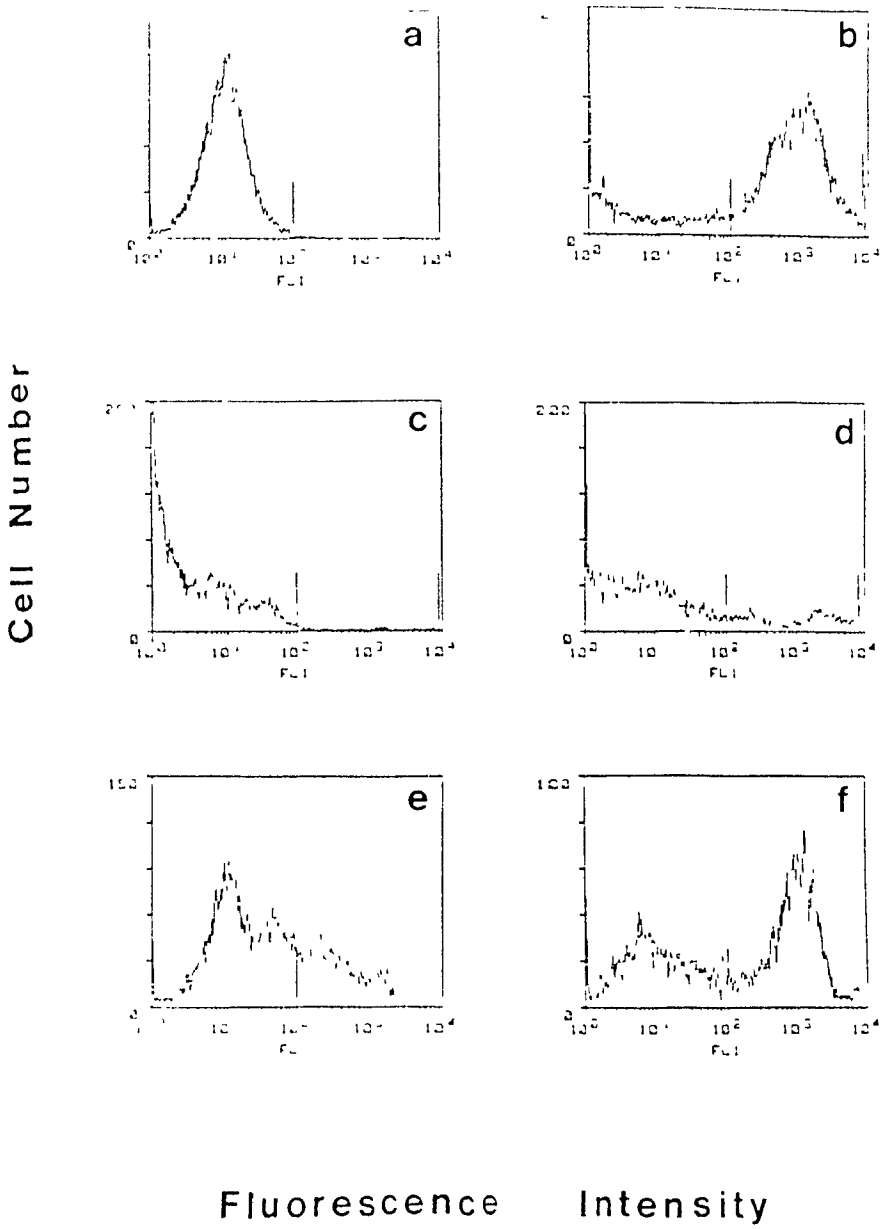

Fig. 6. Immunofluorescence analysis of rat bone-marrow stromal cells with antibodies to vWF. The first antibody was rabbit antibody to vWF and the second TRITC-conjugated swine-anti rabbit antiserum. The profile shown is representative of 4 profiles obtained from FACS analyses. (a) Fibroblasts. (b) human umbilical vein derived endothelial cells (HUVE) (c) BMSC incubated with the second antibody only. (d) BMEC incubated with the second antibody only. (e) BMSC labeled with antibodies to vWF. (f) BMEC labeled with antibodies to vWF.

In phase contrast microscopy these cells exhibited a cobble stone morphology typical of endothelial cells, while BM stromal cells grown in the absence of ECGF were mainly fibroblast like in appearnce (Fig 7).

Adhesion of Mat-LyLu cells to primary cultures of BM stromal cells and to osteoblasts, fibroblasts and liver-derived endothelial cells was subsequently measured. The results shown in Fig 8 demonstrate that the adhesion to BM cultures enriched for endothelial cells (BMEC) was significantly higer than the adhesion to primary cultures of fibroblasts (5.5x), non-enriched, BM-derived stromal cells (3x) and to a lesser extent osteoblasts (1.4x). It was also significantly higher than the adhesion to rat liver sinusoidal endothelial cells (7x)

To determine, whether adhesion to BMEC correlated with metastatic potential in the Mat-LyLu model, the adhesion of Mat-LyLu-B5 cells was measured and compared to the adhesion of the parental line We found that the adhesion of Mat-LyLu-B5 cells to BMEC but not to hepatic EC, fibroblasts or osteoblasts was significantly increased compared to the parent line (Fig. 8).

Finally, to check whether subendothelial matrix proteins playing any role in adhesion of BMEC, we then compared the adhesion of Mat-LyLu-P and Mat-LyLu-B cells to several ECM proteins. The results shown in Fig. 9 demonstrated that Mat-LyLu-B was significantly more adherent than Mat-LyLu-P to fibronectin and vitronectin but not to other ECM proteins tested.

4.5 Discussion

Despite the prevalence of BM metastases in the pathology of human prostate

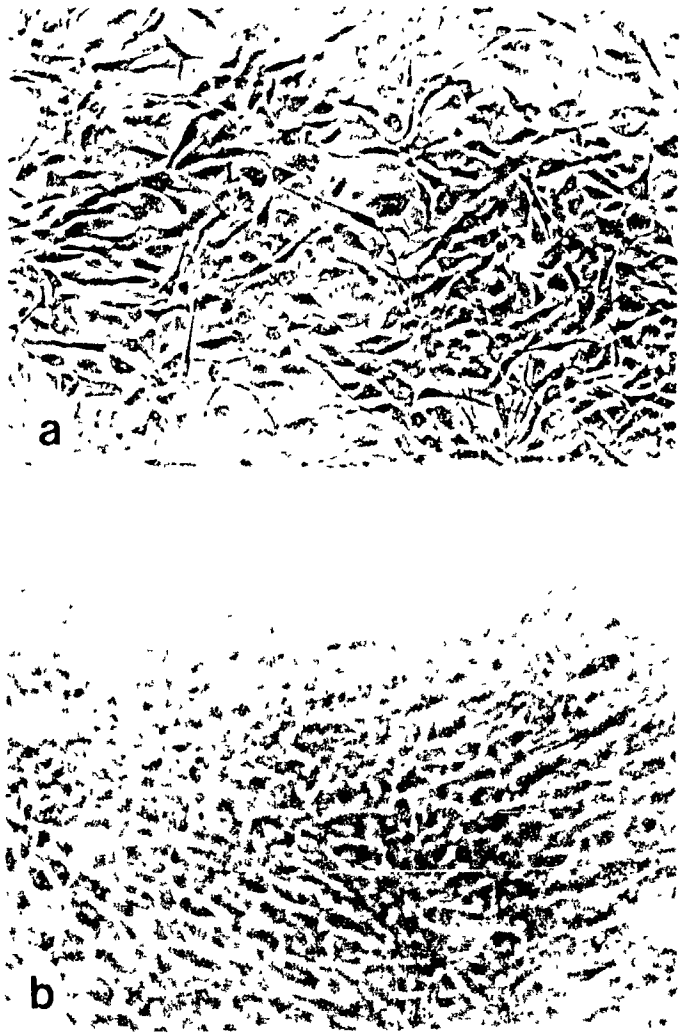

Fig. 7. Phase contrast microscopic view of 3 wks old bone marrow stromal cells cultured with or without the ECGF supplement. (a); Cells cultured without the supplement were fibroblast-like in appearance. (b); Cells cultured in the presence of ECGF had a cobblestone morphology typical of endothelial cells. Magnification x 200.

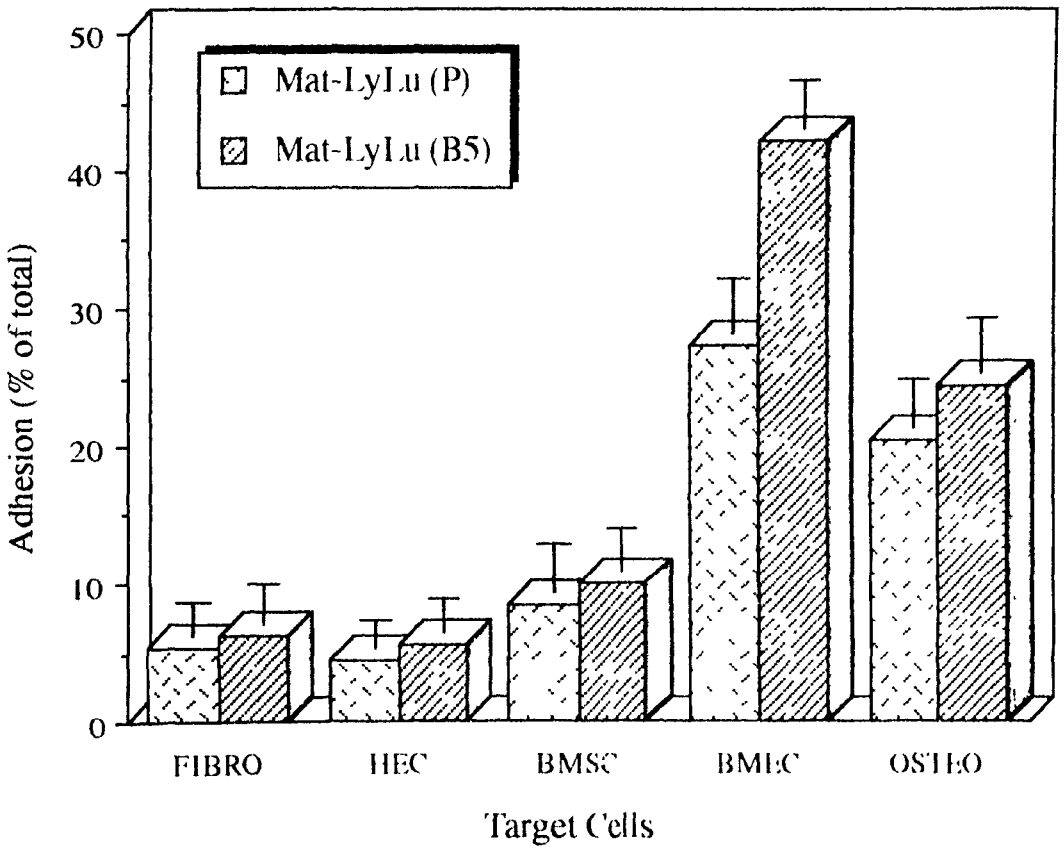

Fig.8. Adhesion of Mat-LyLu cells to cultures of bone marrow stroma highly enriched for endothelial cells correlates with the metastatic potential Results are expressed as mean & S.D. of 3-4 experiments. Adhesion of Mat-LyLu-B5 cells to bone marrow derived endothelial cells (BMEC) but not to hepatic endothelial cells (HEC), fibroblasts (Fibro), osteoblasts (Osteo) and non-enriched stromal cells (BMSC) was significantly increased compared to the parent line ($P < 0.05$). The adhesion of Mat-LyLu-P and Mat-LyLu -B5 cells to BMEC was significantly higher than the adhesion to all other cells tested ($p < 0.05$ for osteoblasts and < 0.005 for other cells).

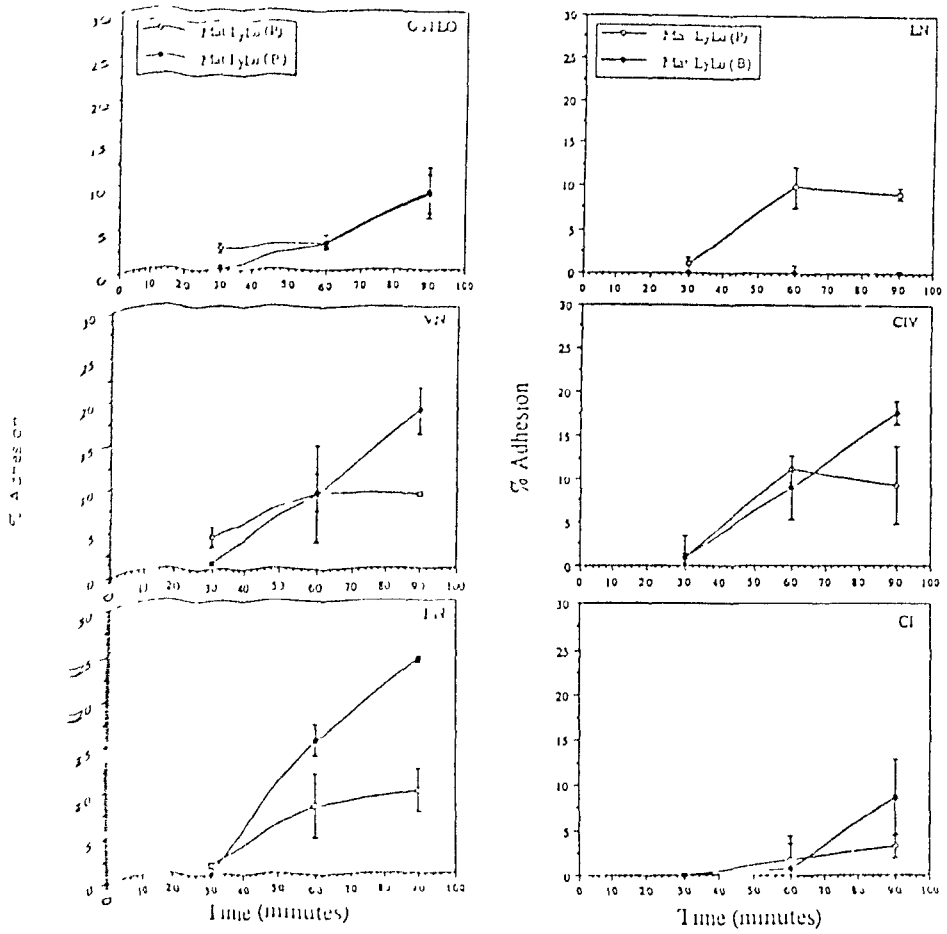

Fig. 9. Adhesion of Mat-LyLu cells to different ECM (extra cellular matrix) proteins. Results are expressed as mean & S.D. of 3-4 experiments. Adhesion of BM metastasizing Mat-LyLu-M cells to Fibronectin (FN) & Vitronectin (VN) but not to Osteopontin (OSTEO), Laminin (LN), Collagen IV (CIV) and Collagen I (CI) was significantly increased compared to parental line ($P < 0.05$).

carcinoma, little progress has been made in understanding the host-tumor interactions underlying this metastatic process. This is due in large measure to the lack of an animal model in which the patterns of dissemination of human prostatic carcinoma can be reproduced.

Prostate carcinoma metastasis to bone has recently been described in two animal models where the intravenous route of inoculation was used in combination with ligation of the inferior vena cava in order to divert the tumor cells to the vertebral venous plexus and away from the lung capillary bed (Shevrin, D.H., et al., 1988; and Geldof, A.A., et al , 1990) Although these models helped to demonstrate that animal prostate carcinoma cells, similarly to the human carcinoma can produce osseous metastases when access to the microcirculation of the bone marrow is provided, they are limited by several drawbacks; (a) the method of inoculation is cumbersome and requires an invasive procedure and (b) the lumbar vertebrae metastases which are produced do not mimic the patterns of bone metastasis seen in human cancer (Arguello, F., et al., 1990).

In the present study metastases were found almost exclusively in the BM following the inoculation of the tumor cells into the left ventricle This occured despite the fact that the total skeletal blood flow represents less than 10% of the cardiac output (Ray, R D , 1976). The patterns of bone colonization, namely the initial growth of the tumor cells in the bone-marrow of the vertebral bodies with subsequent invasion into the spinal canal, closely resembled the pathological picture normally associated with the metastasis of human prostate carcinoma to vertebral bone. It appears therefore that the intra-cardiac route of inoculation provides a means for reproducing more closely the pattern of organ-site specific metastasis characteristic

of metastatic prostate carcinoma.

The reasons for the failure of Mat-LyLu cells to metastasize to bone following s.c. or i.v. inoculation are unclear. It is possible that in the rat, tumor cell arrest in the lung capillaries may bring about death of the majority of cancer cells (Weiss, L., et al., 1986) and/or blockage of their recirculation into the arterial blood flow Alternatively it may lead to alterations in the surface molecules required for adhesion to osseous tissues and BM metastases formation. Since the i.c.inoculation allows the tumor cells to bypass the lung capillary bed, it may result in the release of sufficient numbers of metastatic cancer cells into the circulation and subsequently into the BM vasculature. Therefore, cell access to the target organ vascular bed as well as specific interactions with the organ microenvironment appear to play a role in the patterns of dissemination in the present model. When a similar experiment was carried out with the highly metastatic rat breast carcinoma line TMT-081 (Brodt, p., et al , 1990), no bone metastases were detected 8 weeks after the inoculation of 5×10^4 cells. These findings as well as studies reported by others (Arguello, F., et al , 1991) confirm that vascular access although required is not sufficient for the establishment of BM metastases

The selective adhesion of tumor cells to microvessel endothelial cells derived from the preferred target organ of metastasis has been demonstrated in various tumor systems. Using the B16 melanoma model, Nicolson et al have shown for example, that brain colonizing tumor cells are significantly more adherent to brain derived endothelial cells than lung colonizing B16 cells (Nicolson, G.L., 1982). Auerbach et al. (1987) demonstrated selective attachment between hepatoma cells and liver-derived vascular endothelium, between glioma cells and brain derived endothelial

cells and between teratoma cells and ovary-derived endothelium. Our findings suggest that similarly to vascular endothelium, BM endothelial cells may serve as a site of specific adhesion for bone-homing prostate carcinoma cells

Positive characterization of BM endothelial cells in the present study was on the basis of the two markers Ac-LDL and vWF Although DiI-Ac-LDL uptake is also characteristic of macrophages (Goldstein, J L, 1979), results of latex particle ingestion studies (not shown) indicated that only approximately 3% of the cells in the 3 wk old BMEC cultures were phagocytic, suggesting that the great majority of the Ac-LDL(+) cells were endothelial.

Our results suggest that BM-derived endothelial cells express adhesion ligands for prostatic cancer cells which are not expressed on hepatic endothelial cells or on non-endothelial cell of the bone marrow. This is in accordance with other studies in which the expression of organ-specific ligands on the vascular endothelium has been documented (Auerbach, R., et al., 1987). Extracellular matrix proteins underlying the endothelium have been implicated in the regulation of expression of such ligands (Pauli, B.U., et al., 1988), but the mechanisms involved are not presently clear.

To test whether attachment to the subendothelial matrix proteins is involved in the adhesion to BMEC we recently compared the adhesion of Mat-LyLu-P and Mat-LyLu-B cells to various isolated ECM proteins Results (Fig 9) of these adhesion assays revealed that Mat-LyLu-B cells were significantly more adherent than Mat-LyLu-P cells to Fibronectin (FN) and vitronectin (VN) but not to laminin, osteopontin, and types I and IV collagen However the proportions of cells which adhered to FN and VN at 90 min incubation were 15% and 18% respectively. As adhesion to BMEC reached 43% during the same time interval, it appears that cell-cell rather than cell-matrix

interactions mediated the adhesion to BMEC. It should be noted in this context that a relatively high level of adhesion of Mat-LyLu cells was also seen on cultured osteoblasts (26% adhesion as compared to 44% on BMEC) This suggests that the adhesion ligands recognized by the carcinoma cells on BM endothelium may represent organ-specific determinents also expressed on other bone cells. The osteoblastic response seen in BM colonized by the Mat-LyLu cells and similar osteoblastic metastases associated with human prostate carcinoma metastases also imply a close interaction between these tumor cells and osteoblasts (Koutsilieris, M., et al , 1986).

Adhesion of hematopoietic stem cells to BM stromal cells, including stromal cells expressing endothelial cell markers has been shown to play a central role in hemopoiesis, leading to proliferation and differentiation of the stem cells (Kincade, P.W., et al , 1989). The lymphoid cell surface receptor CD44 which appears to mediate adhesion to the proteoglycan hyaluronate, as well as EGF receptors associated with the stromal cells have been implicated in this adhesive interaction (Miyake, K., et al., 1990) Studies with other tumor models have already shown that adhesion receptors of blood cells may be expressed on tumor cells of diverse origin and mediate tumor cell adhesion and metastasis (Edgar, R. G., et al., and Roossien, F.F., et al , 1989) Recent reports have in fact implicated a variant of the CD44 receptor in tumor metastasis (Gunthert, U., et al., 1991). Consequently the possibility exists that adhesion of prostatic cancer cells to bone marrow stomal cells may involve adhesion ligands for hematopoietic cells. We are currently exploring this possibility.

While prostate carcinoma cells are generally slow growing in the primary site, their growth rate is often enhanced in the bone marrow lesions (Berrettoni, B.A., et al.,

1986 and Jacobs, S.C., 1983). This suggests that the microenvironment of the bone marrow may be a rich source of growth promoting factors for the prostatic cells. In a recent study, Zetter et al have shown that the growth of human prostate carcinoma PC-3 cells can be stimulated by conditioned medium derived from cultured stromal cells of bovine, rat or human BM (Zetter, B R., et al., 1989). The source of the growth stimulating factor(s) is not as yet known. In light of our present results and in view of the studies which link adhesion and proliferation during hemopoiesis in the bone marrow, it is conceivable that prostate carcinoma cell adhesion to the BM endothelium may serve to augment the release of, and response to, growth promoting cytokines secreted by endothelial and\or other stromal cells of the bone marrow (Kincade, P.W , et al., 1989). This hypothesis is the subject of our current investigations.

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CHAPTER V

Summary, Conclusions and Suggestions For Future Studies

5.1 Summary and Conclusions

An experimental model for skeletal metastasis of prostate carcinoma has been developed using the rat Dunning carcinoma line R3327-Mat-LyLu The model consists of two cell lines with divergent potentials to form lumbar vertebrae metastases following the intracardiac injection of the tumor cells into the left ventricle of syngeneic rats. Adhesion studies have shown that the Mat-LyLu cells adhered to cultures of bone marrow (BM)-derived endothelial cells significantly better than to non-endothelial BM stromal cells, osteoblasts or primary cultures of fibroblasts. Moreover, adhesion to the BM endothelium was site-specific as attachment to rat liver-derived endothelial cells was relatively poor. Mat-LyLu cells selected through 5 consecutive intra-cardiac injections of bone metastasizing cells (Mat-LyLu-B5) were significantly more metastatic to bone than the parent line (Mat-LyLu-P). They were also found to be more adherent to BM endothelial cells A significant increase in adhesion to fibronectin and vitronectin compared to laminin, osteopontin, collagen type I and type IV was also observed.

Although the predilection of prostatic carcinoma for the bone provides a unique system for analysis of host and tumor-associated factors which regulate the site-specificity of metastasis, progress in the understanding of the biology of this phenomenon has been slow. This is due mainly to the lack of appropriate animal models. While several prostate carcinoma cell lines are available for study they are not optimal models as they either do not form bone metastases in vivo (Isaacs, J.T., et al., 1986) or require cumbersome methods of inoculation for production of bone metastases (Shevrin, D.H., et al., 1988 and Geldof, A A., et al., 1990) Nude mice

have been used by several investigators to obtain bone metastasis of human prostate carcinoma cells One of the major limitations of this approach has been that the carcinoma cells do not accurately mimic the patterns of metastasis of human carcinoma in nude mice (Shevrin, D.H , et al., 1988). Moreover, Xenogenic models are not ideal for the study of host-dependent factors which may be species specific. The rat model of bone metastasis by Geldof, A.A., et al., (1990) also lacks in reproducing the exact patterns of bone metastasis seen in the human counterpart (Arguello, F., et al., 1990). Ideally, an experimental model for the study of skeletal metastasis of prostatic carcinoma should consist of a histocompatible tumor-host system where the tumor reproducibly gives rise to skeletal metastases in the host, where these metastases reproduce the patterns of growth of human osseous metastases and where sublines with divergent but stable metastatic phenotypes are available for comparative studies.

The metastases observed in our model mimicked closely the patterns seen in the pathology of disseminating human prostatic carcinoma, namely both osteoblastic and osteolytic-type lesions could be seen. Therefore, our model provides an opportunity for in depth analysis of the factors involved in the predilection of prostate carcinoma for bone.

The reasons for the failure of Mat-LyLu cells to metastasize to bone following s.c. or i.v. inoculation are not clear. It is possible that in the rat, tumor cell arrest in the lung capillaries may bring about death of the majority of cancer cells (Weiss, L., and Dimitrov, D S., 1986) and /or blockage of their recirculation into the arterial blood flow. Alternatively, it may lead to alterations in the surface molecules required for adhesion to osseous tissues and BM metastases formation. Since the i.c. inoculation

allows the tumor cells to bypass the lung capillary bed, it may result in the release of sufficient numbers of metastatic cancer cells into the circulation and subsequently into the BM vasculature. When a similar experiment was carried out with the highly metastatic rat breast carcinoma line TMT-081 (Brodt, P., et al, 1990), no bone metastases were detected These finding, as well as studies reported by others (Arguello, F., et al., 1991), confirm that vascular access, although required, is not sufficient for the establishment of BM metastases.

In summary, it appears therefore that in the present model cell access (Mechanical theory) to the target organ vascular bed, as well as specific interactions between the tumor cells and the organ microenvironment (" Seed and Soil" theory) play a role in tumor cell metastasis to bone.

5.2 Suggestions for Future Studies

1. Several adhesion receptors have been described which are involved in the attachment of blood cells to the endothelium and some have also been implicated in cancer dissemination They include ELAM-1, ICAM, VCAM-4, GMP-140, AND PECAM (Simons, D.L, et al, 1990) Their role in prostate carcinoma adhesion could be investigated using the appropriate neutralizing antibodies.

2. If the BM endothelial cells adhesion receptor appears to be a new receptor,

mouse monoclonal anibodies could be generated to the bone marrow endothelial cells as means of identifying unique adhesion receptors. Hybridomas produced following immunization with the endothelial cells can be screened on the basis of their ability to block the adhesion.

3. Urinary plasminogen activator urokinase (uPA) has been associated with malignancies and is believed to play a major role in tumor invasion and metastasis. Its synthesis in prostatic tissue is well documented. The role of uPA and uPA receptor in the increased metastatic potential of Mat-LyLu-B5 should be investigated in the present model Similarly, the role of uPA in the induction of osteoblastic lesions in vivo can be studied. Cell transfection with uPA cDNA can be used as a tool to generate high producer cells and the ability of these cells to from bone metastases and to induce an osteoblastic response can then be assessed.

4. The efficacy of anti-uPA therapy in the inhibition of osseous metastasis in the present model should be of great interest.

These future studies should provide new information on the possible link between adhesion, proteolysis and growth promotion in prostatic carcinoma metastasis to bone. A better understanding of these mechanisms is essential for the development of better prognostic criteria for disseminated prostate carcinoma. More importantly they are expected to form the basis for the development of new therapeutic approaches to the treatment of metastatic prostate carcinoma.

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