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Suppression of the Integrin α_v Subunit by Antisense Oligonucleotides: A Potential Approach for Anti-Angiogenic Therapy

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Abstract

Angiogenesis is essential for reproduction, inflammation, development and wound repair. During these physiological processes, angiogenesis is tightly regulated. However, many pathological diseases, such as tumor growth and metastases, are driven by persistent deregulated angiogenesis. The integrin vitronectin receptor $\alpha_v\beta_3$ has been shown to mediate endothelial cell migration and proliferation and thus, plays a key role in angiogenesis. In the present study, the effect of α_v antisense phosphorothioate oligodeoxynucleotides (ODN) on $\alpha_v\beta_3$ expression, and on cellular migration and proliferation was assessed using human umbilical vein endothelial cells (HUVEC). We found that α_v antisense phosphorothioate ODN reduced $\alpha_v\beta_3$ expression in some endothelial cell cultures and this resulted in a dose-dependent decrease in endothelial cell migration and proliferation. These results suggest that α_v antisense phosphorothioate ODN could potentially be used as a novel class of angiogenesis inhibitors.

Résume

La formation des vaisseaux sanguins joue un rôle vital en vue de la reproduction, inflammation, le development et la guérison des plaies. Pendant ces procèdes physiologiques, la formation des vaisseaux sanguins est régularisée avec beaucoup de précision. Cependant, certains états pathologiques telles les tumeurs et les métastases, sont continuellement entretenues par la formation deregularisee des vaisseaux sanguins. Il a été démontre que l'integrin $\alpha_v\beta_3$ intervient dans la migration et la prolifération des cellules endothéliales et par conséquent, il joue un rôle principal dans la formation des vaisseaux sanguins. Dans l'étude présente, l'effet de l'α_v antisense phosphorothioate ODN sur l'expression de l'$\alpha_v\beta_3$ ainsi que sur la migration et la prolifération cellulaire a été évalue en utilisant des cellules dérivant des veines ombilicales humaines (HUVEC). Nous avons constate que l'α_v antisense phosphorothioate ODN diminue l'expression de l'$\alpha_v\beta_3$ dans certaines cultures de cellules endothéliales. Ceci a abouti à une diminution de la migration et prolifération des cellules endothéliales, dépendamment des doses. Ces résultats suggèrent que l'α_v antisense phosphorothioate ODN pourraient possiblement être utiliser comme une nouvelle catégorie d'inhibiteurs de la formation des vaisseaux sanguins.

Chapter 1

Literature Review

1.1 Angiogenesis

Angiogenesis, the growth of new blood vessels from pre-existing ones, is a process essential in normal physiological conditions including embryonic development (Wagner 1980), reproduction, placental development, inflammation, tissue remodeling and wound healing (Knighton 1981). Under these biological conditions, angiogenesis is a transient and highly regulated process. However, in pathological conditions, persistent deregulated angiogenesis occur as a result of increased production of angiogenic stimulators and decreased production of the negative regulators. This persistent and uncontrolled angiogenesis contributes to the pathogenesis of a variety of diseases such as diabetic retinopathy, rheumatoid arthritis (Folkman 1980a), and chronic inflammation (Polverini 1977), and plays a crucial role in the progressive growth and metastatic spread of tumors (Folkman 1972, 1976b, Maiorana 1978).

Angiogenesis is a multi-step process which involves an orderly sequence of events: (1) the basement membrane of the parent vessel is disrupted and endothelial cell processes penetrate through it into the perivascular tissue; (2) the endothelial cells migrate in the perivascular stroma towards the source of the angiogenic stimulus; (3) endothelial cells at the tip of the sprout continue to migrate and the endothelial cells at the mid-section of the growing capillary sprout undergo proliferation; (4) loop formation occurs as individual capillary sprouts anastomose with each other; (5) differentiation of the capillary sprouts is accompanied by capillary lumen formation; (6) blood flow slowly begins and synthesis of new basement membrane follows; (7) and finally, the formation of an entire capillary network.

1.2 Angiogenesis models

To study the phenomenon of angiogenesis, a number of *in vivo* and *in vitro* models have been developed. Initially, transparent chambers have been inserted into the dermis of the rabbit ear, hamster cheek pouch or dorsal skin of the mouse (Algire 1945, Greenblatt 1968, Warren 1966, Peterson 1979) to observe the growth of capillaries toward tumor implants. One disadvantage of these models was that they could not be considered truly quantitative. Subsequently, several new methods have been developed which allow quantitation of angiogenesis and its resolution into its basic steps (Folkman 1987b).

The first method used by many investigators to study angiogenesis is the chick embryo chorioallantoic membrane (CAM) model. It can be used as a semi-quantitative bioassay for angiogenic activity (Ausprunk 1975, Phillips 1979). In brief, test material on a sterile plastic coverslip is placed onto the chorioallantoic membrane of 9-10 day old chick embryos through a window previously made in the shell. The presence of angiogenesis is determined 48 hours later by observing new capillaries converging onto the disc. This remains one of the most commonly used methods for determining angiogenic activity *in vivo*.

The second quantitative method used to study angiogenesis is the rabbit corneal pocket. The rabbit cornea has been used since 1953 to demonstrate inflammatory neovascularization (Cogan 1949, Gimbrone 1974c). By inserting tumor cells into intracorneal pockets, the cornea technique permits the linear measurement of individually

growing capillaries (Gimbrone 1974c). A further improvement on this method has been developed by Langer and Folkman (Langer 1976b, Ausprunk 1977).

After the corneal micropocket technique was developed, it became feasible to substitute soluble tumor extracts for tumor implants. The sustained-release polymer implants were subsequently developed (Langer 1976b, Rhine 1980, Hsieh 1981, Leong 1985). Using such implants allows the slow release of proteins and other macromolecules *in vivo* in a sustained manner over a desired period of time where a concentration gradient is established (Langer 1976b, Murray 1983). This technique became useful for the study of inhibitors and inducers of angiogenesis. For example, with this technology, it was possible to show that a cartilage extract could inhibit tumor angiogenesis in the cornea (Langer 1976a).

Other less commonly used biological test systems include the implantation of test substance in the rabbit iris (Gimbrone 1976b) or rat dorsal fascia (Folkman 1971a, Phillips 1976), and the intradermal or subcutaneous injection of test material in the mouse, followed by examination of the microvasculature at the site of injection (Sidkey 1975).

In all of these experimental systems, neovascularization can be observed in the tissue where the stimulus is placed. A disadvantage of all these biosystems is the possiblity of obtaining a false positive response, that is angiogenesis which has not been induced by the test sample but by the carrier (e.g. slow-release polymers) or by mechanical injury, via an inflammatory reaction. But this inflammatory angiogenesis can be separated from non-inflammatory angiogenesis by the addition of corticosteroids

together or before application of the test sample (Sholley 1978, Olander 1982, Ziche 1982, Bander 1982).

In vitro, studies of angiogenesis usually employ cultures of large vessel and capillary endothelial cells. This required developing methods for isolation and maintenance of endothelial cells in long-term cultures (Jaffe 1972a, Gimbrone 1974a, Buonassisi 1973, Folkman 1979). Experiments employing capillary endothelial cells demonstrated that all the information neccessary to construct a capillary tube, to form branches, and to build an entire capillary network *in vitro* can be expressed by a single cell type, that is the vascular endothelial cells (Folkman 1980b). However, Maciag *et al.* have shown that capillary tube formation is not restricted to capillary endothelial cells and can also be induced with large vessel cells such as umbilical vein endothelium (Maciag 1982).

A breakthrough in the culture and passage of vascular endothelial cells was reported in 1972 by Jaffe and his associates. They have successfully established primary cultures of human umbilical vein endothelial cells which could be identified by a variety of morphologic, immunohistologic, and serologic criteria (Jaffe 1972a, 1972b). Thereafter, endothelial cells from various sources have been successfully isolated and cultured *in vitro* (Gimbrone 1976c, Ryan 1978, Folkman 1979, Davison 1980).

Human umbilical vein endothelial cells (HUVEC) have been used extensively in a number of *in vitro* models of angiogenesis due to their relevance to human disease. There are several advantages to employing human umbilical veins as a source of endothelial cells. Umbilical cords are widely available as opposed to the availability of human microvascular vessels. In addition, the cannulation, flushing and recovery of effluents are

technically uncomplicated. However, although the primary culture of human endothelial cells from umbilical veins is well established (Maciag 1981, Jaffe 1973b), many variables such as the different sources of umbilical cords and the age difference between the placentas, can affect the properties of the isolated cells. In addition, HUVEC have fastidious exogenous growth factor requirements and need special culture substrata (fibronectin, gelatin) (Maciag 1981, 1984a, Jaffe 1973b), cofactors (heparin, ascorbic acid) (Thorton 1983), and high concentrations of serum (Jaffe 1973b) for optimal growth and serial propagation. Even under these optimized growth conditions, the relatively short life-span in culture of HUVEC and their long population doubling time of 92 h (Jaffe 1973b, Takahashi 1990) can become a major obstacle, especially when large quantities of endothelial cells are required for experimental analyses. Furthermore, contamination of endothelial cell cultures by fibroblast and/or smooth muscle cells can often complicate the analysis (Gimbrone 1973). Also, primary HUVEC isolates often exhibit functional instability with increased passage. For instance, with the use of the ^{3}H-thymidine incorporation assay, our laboratory has found that as the passage number of the cell cultures increases, their rate of proliferation decreases. All these factors may cause a discrepancy in results. More studies or characterization need to be done in order to have a better understanding of the nature of HUVEC.

Numerous electron microscopic studies (Cliff 1963, Yamagami 1970, Warren 1972, Ausprunk 1977) as well as light microscopic studies (Williams 1959, Cliff 1963, Warren 1966) confirmed *in vivo* observations and have shown that angiogenesis is a multi-step process that requires an orderly sequence of events. With the better understanding of the angiogenic process and with the establishment of cultured

endothelial cells, it has been possible to develop *in vitro* bioassays based on the three sequential events of capillary growth *in vivo*: (1) enzymatic degradation of the extracellular matrix (Gross 1983), (2) endothelial cell migration (Zetter 1980) and proliferation (Birdwell 1977, Gospodarowicz 1976, Folkman 1979), and (3) endothelial cell differentiation.

Directional locomotion of endothelial cells is an early event in the formation of a capillary and dominates the process. It has been shown that an angiogenic signal can act as a chemotactic trigger and that angiogenic factors such as vascular endothelial growth factor (VEGF), can induce endothelial cells to secrete high concentrations of metalloproteinases (MMP-1, MMP-2, MMP-9) and plasminogen activators (uPA), which can degrade the basement membrane (Moscatelli 1981, Gross 1983) and facilitate cell migration through matrix barriers.

Endothelial cell migration, which is a key component of neovascularization *in vivo* (Ausprunk 1977), can also be demonstrated *in vitro*. Cell migration *in vitro* has been studied using several assay systems with cultured endothelial cells (a) by measuring the radial expansion of a colony in a punch hole made in an agarose gel (Wall 1978, Thorgeirsson 1979), (b) by producing a "wound" in a confluent monolayer and counting the number of cells migrating from the edge of the wound to fill the gap (Sholley1977), (c) by measuring the phagokinetic tracks made by cells plated on glass coverslips coated with colloidal gold (McAuslan 1980, Zetter 1980) and (d) by counting the number of cells that traverse a filter set in a Boyden chamber (Glaser 1980, Castellot 1980). In the Boyden chamber assay, chemotaxis (i.e. directed migration) is induced by introducing

different concentrations of the test substance (which serves as a chemoattractant) in the two compartments, thereby establishing a concentration gradient across the filter.

Endothelial cell proliferation is another aspect of the angiogenic process. Endothelial cell proliferation has been well examined in *in vitro* experimental systems by estimating (a) the total cell number (Fenselau 1976, Schor 1980), (b) the incorporation of ^{3}H-thymidine into the nucleus during DNA synthesis (Gimbrone 1974a, Fenselau 1981), (c) the number of surviving and/or proliferating cells with the use of the MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. This assay is dependent on the reduction of the MTT reagent by the dehydrogenase enzymes present in active mitochondria of viable cells to a blue formazan product which can be measured spectrophotometrically (Mossmann 1983), (d) the total amount of cellular DNA (Olander 1982), and (e) colony number (Atherton 1977).

1.3 Pro-angiogenesis factors

Under normal physiological conditions, angiogenesis is tightly regulated by a dynamic balance between factors stimulating and inhibiting the process. Among all the angiogenesis-inducing factors identified to date, the best characterized include acidic fibroblast growth factor (aFGF) (Maciag 1984b), basic fibroblast growth factor (bFGF) (Shing 1984), platelet-derived endothelial cell growth factor (PD-ECGF) (Folkman 1992), transforming growth factor-α (TGF-α) (Folkman 1992) and vascular endothelial growth factor (VEGF) (Ferrara 1989).

Table 1. Basic properties and biological activities of angiogenic factors *in vitro*

Factor	Molecular Weight (D)	Source	Stimulates endothelial cell proliferation	Stimulates endothelial cell migration
Acidic FGF	16,400	Neural tissue	Yes	Yes
Basic FGF	18,000	Brain and pituitary gland	Yes	Yes
VEGF	45,000	Tumors, pituitary gland cells	Yes	Yes
TGF-α	5,500	Transformed fibroblasts, tumors, macrophages	Yes	Not determined
TGF-β	25,000	Platelets, bone	Yes	No
PD-ECGF	45,000	Platelets, endothelial cells	Yes	Yes

The identification and purification of these angiogenic factors has been facilitated by the availability of quantitative *in vitro* assays (Folkman 1987a, 1987c). One concept which emerged from these analyses is that not all angiogenic factors have the same effects *in vitro* (Table 1).

Some angiogenic factors have been shown to stimulate endothelial cell migration as well as proliferation (Zetter 1980, Castellot 1982, Suddith 1975, Olander 1982). Of these, the best studied are bFGF and VEGF.

The first complete purification of an endothelial cell growth factor was based on heparin affinity chromatography (Shing 1984, 1985) of a rat chondrosarcoma-derived protein. When the protein was subsequently sequenced (Esch 1985), it was identified as basic fibroblast growth factor (bFGF). bFGF is a 146-amino acid polypeptide whose molecular weight is 18,000Daltons (Esch 1985). In cells expressing bFGF such as pituitary gland, cartilage, chondrosarcoma, and hepatoma cells, two mRNA transcripts of 5 and 2.2-kb were detected (Abraham 1986). Amino acid sequence analysis indicated that bFGF is synthesized as a 154-amino acid polypeptide and subsequently cleaved at the amino-end to yield a truncated protein of 146-amino acid residues (Ueno 1986, Burgess 1985).

bFGF is a potent stimulator of endothelial cell proliferation (Shing 1984) and motility (Biro 1994). The role of bFGF in angiogenesis was confirmed using chick embryo bioassays (Shing 1985, Esch 1985, Lobb 1985), rat corneal bioassays (Shing 1985, Lobb 1985) and sponge implantations in the rat (Davidson 1986). In addition, during the process of angiogenesis, four bFGF-related high-affinity tyrosine kinase

receptors have been shown to be highly expressed on activated endothelial cells (Klagsbrun 1991), supporting the role of this factor in angiogenesis.

In normal and some pre-malignant cells, bFGF is not secreted in a soluble form but stored in the basement membrane (Abraham 1986). This cell-associated angiogenic factor is released only under restricted physiological conditions where neovascularization is required. In contrast to its inactivity in normal tissues, tumor cells can initiate bFGF-mediated angiogenesis by several mechanisms including the elaboration of hydrolytic enzymes that can mobilize bFGF from the extracellular matrix stores. Among the proteolytic enzymes which can release latent bFGF are heparanases, collagenases and plasminogen activators, all of which have been implicated in the metastatic process (Vlodavsky 1990). The angiogenic potential of bFGF has been utilized in several clinical conditions where acceleration of neovascularization is desired including the treatment of chronic wounds (Robson 1992) and duodenal ulcers (Folkman 1991).

VEGF is a member of a family of related factors. Recently, four VEGF-related genes have been identified namely VEGF-B (Olofsson 1996), VEGF-C (Joukov 1996), VEGF-D (Yamada 1997) and placenta growth factor (Maglone 1991). The gene products of these related genes are glycosylated and secreted as dimers cleavage of their signal peptide. VEGF is a highly conserved protein that has cross-species activity. Between the human, rat and bovine VEGF, 84-94% sequence homology was observed (Schott 1993, Neufeld 1994). VEGF has been purified from the conditioned medium of a variety of cell types including bovine folliculostellate cells (Ferrara 1989,Gospodarowicz 1989), tumor cell line AtT-20 derived from mouse anterior pituitary gland (Plouet 1989), and a rat glioma cell line (Conn 1990). Molecular cloning of the cDNA showed that VEGF shares

an overall homology of 18% with platelet-derived endothelial cell growth factor (PD-ECGF) (Tischer 1989). The human VEGF gene is organized into eight exons separated by seven introns (Tischer 1991). As a result of alternative splicing, four transcripts encoding monomeric VEGF consisting of 206 (V206), 189 (V189), 165 (V165), and 145 (V145) amino acid residues each have been identified. The most predominant isoform is V165, a diffusible secreted protein, which generates a 45 kDa peptide upon signal peptide cleavage.

VEGF expression is markedly increased in a variety of tumors such as renal cell carcinomas (Takahashi 1994), human ovarian neoplasms (Olson 1994), adenocarcinomas of the gastrointestinal tract (Brown 1993b), and central nervous system neoplasms (Berkman 1993). Moreover, it has been shown that both VEGF and its endothelial cell receptors (flt-1 and KDR) are commonly overexpressed in tumor-associated endothelial cells in comparison with the vasculature of the surrounding tumor-free tissue (Senger 1983, Plate 1992). VEGF and its endothelial cell receptors are also overexpressed in other pathological conditions that are characterized by angiogenesis such as healing of cutaneous wounds (Brown 1992), psoriasis (Detmar 1994), delayed-type hypersensitivity (Brown 1995a), myocardial ischemia (Ladoux 1993, Sharma 1992) and rheumatoid arthritis (Koch 1994, Fava 1994). VEGF and its receptors are also upregulated in physiological processes involving neovascularization such as reproduction, the menstrual cycle and embryogenesis (Brier 1992, Jakeman 1993, Ravindranath 1992).

Unlike other angiogenic growth factors, VEGF is devoid of appreciable mitogenic activity for other cell types (e.g. epithelial cells, keratinocytes or fibroblasts) (Ferrara 1989, 1992, Plouet 1989). Similarly to bFGF, VEGF can stimulate endothelial cell

division (Ferrara 1992, Gospodarowicz 1989, Houck 1991, Sioussat 1993). It can also induce degradation of the subendothelial basement membrane. This is mediated through upregulation of plasminogen activators (PA), such as urokinase plasminogen activator (uPA), which have been implicated in the metastatic process (Pepper 1991). VEGF also induces the expression of the metalloproteinase interstitial (type I) collagenase (Unemori 1992). The induction of both plasminogen activators and collagenase by VEGF provides the necessary conditions for degradation of the extracellular matrix and subsequent migration of endothelial cells (Favard 1991, Koch 1994). VEGF can also upregulate expression of the integrin $\alpha_v\beta_3$ which promotes endothelial cell migration on substrata consisting of $\alpha_v\beta_3$ ligands such as vitronectin (Senger 1996). Finally, VEGF has been shown to promote angiogenesis in many *in vitro* and *in vivo* assay systems (Connolly 1989a, Wilting 1992, Ferrara 1992). More direct evidence that VEGF plays a role in tumor angiogenesis have been derived from studies using specific monoclonal antibodies which inhibit VEGF-induced angiogenesis *in vitro* and *in vivo* (Kim 1993).

VEGF is also known as vascular permeability factor (VPF) because of its vessel permeabilizing activity (Senger 1993, Collins 1993, Connolly 1989b). VEGF increases microvascular permeability by enhancing the activity of the vesicular-vacuolar organelle (VVO) (Kohn 1992, Qu-Hong 1995). VVOs provide a pathway whereby macromolecules such as plasma proteins, may exit the circulation and enter the tissues (Rippe 1994). In normal tissues, extravasation of circulating macromolecules from the microvasculature is quite limited and takes place by way of VVOs (Kohn 1992). In tumor vessels, VVO function is substantially upregulated. This is supported by the finding that tumor-

supplying microvessels become 4- to 10-fold more permeable than their normal tissue counterparts (Dvorak 1979a, 1984b, 1988).

Published data from a number of different laboratories indicate that microvascular hyperpermeability regularly accompanies and likely has a mechanistic role in the induction of angiogenesis (Dvorak 1992, Heuser 1986, Nagy 1995). Plasma proteins such as fibrinogen, fibronectin and albumin, extravasate from leaky blood vessels that supply tumors and form a new provisional matrix that allows and favors the inward migration of endothelial cells and fibroblasts (Dvorak 1983, 1986). A large body of evidence suggest that migrating endothelial cells and stromal fibroblasts act synergistically to form new blood vessels. The fibroblasts synthesize and secrete the matrix proteins, proteoglycans, and glycosaminoglycans that make up the mature tumor stroma (Dvorak 1986, Yeo 1995). Moreover, plasma fibrinogen that extravasates at tumor sites clots to form crosslinked fibrin (Dvorak 1985, Brown 1988b). Fibrin exerts its pro-angiogenic effect by providing a matrix favorable for cell adhesion and migration, presumably via its Arg-Gly-Asp (RGD) sequence (Brown 1993c). This is strongly supported by direct evidence from both *in vitro* (Lanir 1988, Brown 1993c) and *in vivo* studies (Dvorak 1986, 1987). Brown *et al.* developed a quantitative *in vitro* assay that permits the study of fibroblast migration in fibrin gels. They discovered that the extent of fibroblast migration was greatly affected by the nature of the fibrin clot. Migration was greatly enhanced by extensive cross-linking of the fibrin alpha-chains by factor XIIIa. When alpha-chain cross-linking was inhibited, fibroblasts migrated poorly. Further purification of fibrinogen by anion exchange high pressure liquid chromatography demonstrated that purified fibrinogen clotted to form fibrin gels induces fibroblast migration. In addition,

Dvorak and colleagues implanted subcutaneously, into guinea pigs, plastic chambers filled with cross-linked fibrin. They demonstrated that cross-linked fibrin induced an angiogenic response. However, chambers filled with type I collagen or agarose did not induce new blood vessel formation. These findings provide evidence for the critical role of fibrin or related proteins in angiogenesis. Other circulating RGD-containing plasma proteins that extravasate from leaky blood vessels at sites of tumor growth and contribute to the generation of an extracellular matrix favoring angiogenesis and the formation of new stroma include fibronectin and vitronectin.

Transforming growth factors (TGF) were originally isolated from virally-transformed rodent fibroblasts (Todaro 1980, Mizel 1980). Two structurally distinct TGFs, TGF-α and TGF-β have been purified. TGF-α, a 50-amino acid polypeptide with a molecular weight of 5,500 D, is synthesized by a variety of transformed cells (Schreiber 1986), tumor cells, as well as normal cells such as macrophages (Madtes 1988, Rappolee 1988). *In vitro*, TGF-α stimulates microvascular endothelial cell proliferation (Schreiber 1986) and its potent angiogenic activity was demonstrated *in vivo* in the hamster cheek pouch model (Derynck 1990).

Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45,000 D polypeptide which stimulates DNA synthesis in endothelial cells and also acts as a chemotactic factor. It was reported to induce synthesis of fibroblast growth factors (FGF) in endothelial cells (Ishikawa 1989).

1.4 The contribution of angiogenesis to cancer

Tumorigenesis is a multi-step process that begins with the loss of normal control of cell proliferation. Gullino and associates have demonstrated that during the multi-step process of transformation from a normal cell to one that is neoplastic, angiogenesis precedes tumorigenesis and the onset of angiogenic activity is now recognized as another discrete step in tumorigenesis. This phenomenon was seen in experiments with mouse and rat mammary carcinomas (Gimbrone 1976a, Brem 1977, Maiorana 1978), in human breast carcinoma (Brem 1978, Chodak 1981) and in carcinogenesis where plastic discs are implanted subcutaneously (Ziche 1981). Based on the knowledge that large plastic coverslips implanted subcutaneously in inbred CBA mice (Whitmore 1985) can produce sarcomas more rapidly and in a greater number than do small coverslips, Ziche *et al.* (Ziche 1981) demonstrated that a few weeks after implantation, the cells attached to the large coverslips showed an angiogenic capacity about 5-fold greater than that of the cells attached to the small coverslips. Months before a sarcoma was evident, angiogenesis induced by the cells attached to the large coverslips predicted the high risk of neoplastic transformation by large coverslips. Many studies have shown that tumor growth is angiogenesis-dependent (Folkman 1973, Gimbrone 1972, Tannock 1970).

The first strong evidence demonstrating that tumor growth is accompanied by new capillary growth emerged in 1939, from studies of tumors implanted in transparent chambers in the rabbit ear (Ide 1939). In 1945, based on investigations of similar transparent chambers implanted in mice, Glen Algire and his colleagues demonstrated that the rapid and progressive growth of a tumor is dependent upon the development of a rich vascular supply. They also concluded that the tumor itself is responsible for continuously eliciting the ingrowth of new blood vessels from the surrounding tissue, a

process referred to as tumor-induced angiogenesis (Algire 1945). Further evidence demonstrating that tumor growth is dependent on neovascularization came from the growth of tumors implanted into organs maintained in isolated perfusion glass chambers (Folkman 1964, 1966). From these experiments and those of Algire (Algire 1945), Folkman proposed a hypothesis that all tumors are angiogenesis-dependent (Folkman 1972). Folkman also postulated that once a tumor is established, expansion of tumor cell population beyond the initial pre-vascular or *in situ* stage must be preceded by an increase in new capillaries that converge upon the tumor (Folkman 1976a, 1984). This hypothesis implied that angiogenesis is a control point common to most solid tumors and that angiogenesis is critical for tumor growth, as new blood vessels supply the tumor with oxygen, nutrients, and growth factors.

As summarized above, a number of soluble factors, derived from both neoplastic and non-neoplastic tissues, have been identified as inducers of angiogenesis (Folkman 1987b). This led to the realization that the difference between angiogenesis induced by neoplastic and non-neoplastic tissues may be quantitative rather than qualitative in nature. While expression of endothelial cell growth factors is tightly regulated in normal cells and is induced only under specific conditions such as wound healing, tumor cells constituitively express high levels of endothelial cell growth factors. Therefore, a temporal difference in the expression of endothelial growth factors is the major distinction between normal angiogenesis and tumor-induced angiogenesis. In addition to tumor cell-derived angiogenic factors (Folkman 1971a), tumor-induced angiogenesis can also be stimulated by host inflammatory cells which infiltrate the tumor site such as activated macrophages (Polverini 1984), and mast cells (Dethlefesen 1990). Mast cells

release heparin, which can potentiate angiogenesis by stabilizing basic fibroblast growth factor (bFGF) - a heparin-binding angiogenic factor.

Studies have also demonstrated that angiogenesis is a critical component of tumor metastasis. Following the establishment of a blood supply, a dormant tumor will begin to grow (Greene 1941, Folkman 1974) and has the potential of being disseminated to distant sites via the haematogenous route (Warren 1976). Angiogenesis facilitates tumor metastasis by providing an increased density of highly permeable blood vessels that have little basement membrane and fewer intercellular junctional complexes than normal blood vessels. This serves as an efficient route of exit for tumor cells to shed from the primary site into the circulation and ultimately spread to other organs throughout the body. Because the number of metastases is likely to be proportional to the number of cells in the circulation (Folkman 1971b, 1974), a decrease in angiogenesis at the primary site could lead to a reduction in the number of tumor cells which access the systemic circulation and as a result, decrease the number of metastatic colonies in distant target organs. For these reasons, the process of metastasis is considered to be angiogenesis dependent (Folkman 1971b, 1974).

1.5 Role of cellular adhesion in angiogenesis

During the process of angiogenesis, endothelial cells adhere to extracellular matrix proteins. Extracellular matrix proteins have been shown to regulate not only endothelial cell attachment and spreading, but also their motility and their response to growth and transforming factors. Experimental evidence suggests that appropriate

interactions of endothelial cells with the substratum are critical in the process of angiogenesis. The induction of tubes observed by embedding bFGF-stimulated endothelial cells within a three-dimensional collagen gel, or in a sandwich between two gels, supports the hypothesis that interactions between endothelial cells and extracellular matrix proteins are required to induce angiogenesis *in vitro* (Liu 1990, Madri 1988, Montesano 1986).

Cell adhesion is mediated by four major families of adhesion molecules: the integrins, members of the immunoglobulin (Ig) supergene family, selectins, and cadherins. Integrins represent the largest known family of cell adhesion molecules, and are expressed by many different cell types including epithelial cells, platelets, leukocytes, and mesenchymal tissues (Hynes 1992). Integrins have also been identified as cell surface receptors on endothelial cells (Defilippi 1991). Integrins are transmembrane adhesion receptors that mediate cell-extracellular matrix interactions, such as cell adhesion and migration. Some integrins also induce blood vessel formation by mediating cell-cell adhesion. For instance, vascular adhesion molecule 1 (VCAM-1) which is a member of the Ig supergene family, has been shown to induce migration of human endothelial cells *in vitro* and this induction was significantly inhibited by antibodies against the integrin $\alpha_4\beta_1$ (Koch 1995). This implies that VCAM-1 plays a role in the process of angiogenesis through its interaction with integrin $\alpha_4\beta_1$ (Stromblad 1996b).

Integrins are heterodimers consisting of non-covalently associated α and β subunits (Ruoslahti 1991, Hynes 1992). The interaction of integrins with the cytoskeleton and the extracellular matrix requires the participation of both α and β subunits (Buck

Table 2. The integrin receptor family and their ligands

Subunits		Ligands
β_1	α_1	Collagens, laminin
	α_2	Collagens, laminin
	α_3	Fibronectin, laminin, collagens
	α_4	Fibronectin, VCAM-1
	α_5	Fibronectin (RGD)
	α_6	Laminin
	α_7	Laminin
	α_8	?
	α_v	Vitronectin, fibronectin
β_2	α_L	ICAM-1, ICAM-2
	α_M	C3b component of complement (inactivated), fibrinogen, factor X, ICAM-1
	α_X	Fibrinogen, C3b component of complement (inactivated)
β_3	α_{IIb}	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin
	α_v	Vitronectin, fibrinogen, von Willebrand factor, fibronectin, thrombospondin, osteopontin, collagens
β_4	α_6	Laminin
β_5	α_v	Vitronectin, HIV Tat protein
β_6	α_v	Fibronectin, tenascin
β_7	α_4	Fibronectin, VCAM-1
	α_E	E-cadherin
β_8	α_v	?

1986). Combinations of different integrin subunits on the cell surface allow cells to recognize and respond to a variety of different extracellular matrix proteins (Table 2). However, individual integrins may bind to more than one ligand. Equally, individual ligands can be recognized by more than one integrin. There are eight known β subunits and fourteen known α subunits. Although these eight β subunits and fourteen α subunits could in theory associate to yield more than a hundred integrin heterodimers, the actual diversity is more restricted. Many α subunits can associate with only a single β subunit, while several others (e.g. α_v, α_6, α_4) can associate with more than one β subunit. The α_v subunit is particularly promiscuous in this respect as it can associate with the β_1, β_3 and β_5 subunits forming integrin receptors with different ligand specificities (Albelda 1990, Horton 1990).

The importance of the α_v integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, in angiogenesis has been demonstrated by numerous studies (Brooks 1994b, 1994c, Friedlander 1995). Integrin $\alpha_v\beta_3$ recognizes the Arg-Gly-Asp (RGD) sequence found within a number of extracellular matrix proteins including fibronectin, vitronectin, type I collagen, denatured type IV collagen (a.k.a. gelatin), Von Willibrand's factor, osteopontin, and adenovirus penton base (Cheresh 1993, Leavesley 1992, Wickham 1993). Although the expression of the integrin $\alpha_v\beta_3$ in normal quiescent endothelial cells is low, it has been demonstrated that it is upregulated on proliferating endothelial cells in the process of angiogenesis. Upregulation of the $\alpha_v\beta_3$ integrin has been observed in blood vessels in human wound granulation tissue (Brooks 1994b), on blood vessels in pig skin during wound repair (Clark 1996), during angiogenesis induced in the chick chorioallantoic membrane model

(Brooks 1994b), on capillary sprouts in neonatal human foreskin (Enenstein J 1994), and on choroidal and retinal neovascular membranes (Friedlander 1996).

Angiogenesis depends on the stimulation of quiescent endothelial cells by angiogenic factors to express the integrin $\alpha_v\beta_3$ (Brooks 1994b), and also on the interactions of $\alpha_v\beta_3$ with its ligands (Brooks 1994b, 1994c). An NPXY sequence in the cytoplasmic tail of the β_3 subunit has been implicated in the signaling events required for cellular migration (Filardo 1995). In addition, Brooks *et al.* (Brooks 1996) demonstrated that the type IV collagenase (MMP-2) binds directly to integrin $\alpha_v\beta_3$ and is localized on the surface of invasive tumor cells or activated endothelial cells. This localization provides migratory cells with a mechanism for coordinated matrix degradation and cellular motility, thereby facilitating cellular invasion processes (Brooks 1996). The highly restricted expression of $\alpha_v\beta_3$, the upregulation of its expression during angiogenesis, and its ability to mediate endothelial cell proliferation and migration suggest that $\alpha_v\beta_3$ plays a critical role in angiogenesis. Recent experimental evidence supports this notion. Blocking of endothelial $\alpha_v\beta_3$ function with monoclonal antibodies and synthetic peptides was shown to suppress ingrowth of blood vessels to tumor implants in the chicken chorioallantoic membrane model (Brooks 1994c), quail embryo (Drake 1995), rabbit cornea (Friedlander 1995), mouse retina (Hammes 1996), and in human skin transplanted onto SCID (severe combined immunodificiency) mice (Brooks 1995a, Drake 1995, Friedlander 1995, Hammes 1996). Antagonists of integrin $\alpha_v\beta_3$ could also induce tumor regression *in vivo* (Brooks 1994c).

By mediating transmembrane signaling from the extracellular matrix, integrins not only participate in cell proliferation and migration necessary for angiogenesis (Clark 1995, Yamada 1995), but also regulate cell survival. In many cases, these signals have been linked to the regulation of gene expression and contribute to mechanisms such as cell survival. Endothelial cells are anchorage-dependent and undergo apoptosis when denied integrin-mediated attachment. Recent studies have demonstrated that integrin-mediated cell adhesion can protect cells from apoptosis (Brooks 1994a, Frisch 1994, Meredith 1993, Montgomery 1994), indicating that integrins regulate endothelial cell survival *in vitro* (Meredith 1993, Re 1994). It has also been reported that blocking the function of integrin $\alpha_v\beta_3$ results in the induction of apoptosis among proliferating vascular cells (Brooks 1994a). Notably, the mechanism of action of $\alpha_v\beta_3$ antagonists in blocking angiogenesis appears to be related to their ability to selectively promote apoptosis of newly sprouting blood vessels (Brooks 1994a, 1994c, Stromblad 1996a). Integrin $\alpha_v\beta_3$ appears therefore to function as a survival factor as well as a mediator of cellular proliferation and migration during angiogenesis.

In addition to integrins, a number of other cell adhesion molecules have been implicated in angiogenesis. Among them, the most extensively studied are those of the immunoglobulin-like (Ig-like) and selectin families. The Ig-like molecules, also known as the immunoglobulin supergene family, mediate heterophilic or homophilic cell-cell adhesion. Several members of this family have been shown to be highly expressed during angiogenesis. Intercellular adhesion molecule 1 (ICAM-1) expression is low on quiescent endothelial cells; however, during inflammation, their expression is upregulated (Brooks 1996a). Another Ig-like molecule, platelet and endothelial cell-adhesion molecule

(PECAM), is expressed abundantly on both capillaries and large vessel endothelium during tumor-induced angiogenesis (Berger 1993). Endothelial-cell-specific VE-cadherin (Breier 1996, Dejana 1996) has also been proposed to play a role in angiogenesis based on its expression in angiogenic blood vessels and their localization (Berger 1993). As already described, there is also strong evidence that the interaction between the Ig-like molecule vascular adhesion molecule-1 (VCAM-1) and the integrin receptor $\alpha_4\beta_1$ plays a role in angiogenesis (Koch 1995). In its soluble form, VCAM-1 induces angiogenesis *in vivo* and migration of human endothelial cells *in vitro* (Koch 1995).

Several lines of evidence also suggest that E-selectin, a member of the selectin family of adhesion molecules, participates in angiogenesis. For example, antibodies to E-selectin or sialyated glycans - the E-selectin ligands, could inhibit blood vessel formation *in vitro* (Nguyen 1992, 1993, 1996). It has also been demonstrated that E-selectin is highly expressed in proliferating endothelial cells in infantile hemangiomas and other non-inflammatory angiogenic tissues, and that the levels of E-selectin correlate with angiogenesis (Kraling 1996).

1.6 Angiogenesis inhibitors

With the development of synthetic inhibitors of angiogenesis, it was demonstrated that certain animal tumors regress when angiogenesis is inhibited (Langer 1980, Folkman 1975). Anti-metastatic effect was also demonstrated with various angiogenesis inhibitors (see below). These experimental findings were the basis for the development of

angiogenesis inhibitors as a new class of pharmacologic agents to be used in anti-tumor therapy and other diseases involving abnormal neovascularization.

The concept of "anti-angiogenesis" as a potential therapeutic approach was first proposed in 1971 (Folkman 1971b). Anti-angiogenesis therapy generally targets endothelial cells and is aimed at blocking their proliferation and/or migration. Anti-angiogenesis therapy has several advantages. Firstly, endothelial cells are easily accessible from the circulation. Secondly, in contrast to tumor cells which are genetically unstable and which may eventually develop drug resistance, endothelial cells are genetically stable and have a low mutational rate. In fact, the ability to circumvent the problems associated with acquired drug resistance in tumors has been demonstrated with the use of anti-angiogenic agents such as endostatin (see below) (Boehm 1997).

The first tissue identified as a source of angiogenesis inhibitors was cartilage - a normal tissue that is poorly vascularized (Brem 1975, Folkman 1976a). Einenstein and colleagues reported that when cartilage was placed on the chick chorioallantoic membrane, avascular zones formed around the cartilage implants (Eisentein 1973), suggesting that a cartilage-derived factor could block the formation of new vessels. This result was extended by Brem and Folkman (Brem 1975) who showed that cartilage could inhibit the angiogenic response to tumors, and by Langer *et al.* (Langer 1980) who showed that tumors implanted in the mouse cornea failed to become vascularized when the mice were infused with extracts of a cartilage-derived inhibitor. With the purification of this factor, it was found that this factor has a high sequence homology with a class of collagenase inhibitors present in cartilage (Murray 1986). This suggested that

angiogenesis can be blocked by inhibiting the process of matrix degradation mediated by migrating endothelial cells (Kalebic 1983).

A number of endogenous anti-angiogenic factors (Table 3) have since been identified. They include thrombospondin-1 (TSP-1, Good 1991, Bagavandoss 1990, Iruela-Arispe 1991, Grossfeld 1997, O'Reilly 1997), angiostatin (Cao 1997, Sack 1999, Bergers 1999), endostatin (Hayes 1999, Bergers 1999), interferon (Sidky 1987), tissue inhibitors of metalloproteinases (TIMPS) (Johnson 1994, Anand-Apte 1997, Murphy 1993), cytokines (IL-4, IL-2), and proteolytic breakdown products of several proteins including prolactin (Clapp 1997), and collagen XVIII (O'Reilly 1994, 1997). Among them, TSP-1, angiostatin and endostatin are some of the most potent ones.

TSP-1 was originally identified in the alpha granule of platelets. O'Shea and Dixit showed its presence in quiescent vessels and its absence from actively growing blood vessels in the developing mouse embryo (O'Shea 1988). Bouck and colleagues then discovered that there is a correlation between the anti-angiogenic activity of TSP-1 and the expression of a tumor suppressor gene (Rastinejad 1989, Good 1990). This correlation was clarified by the evidence that the tumor suppressor gene p53 could repress angiogenesis by upregulating the production of TSP-1 (Dameron 1994, Van Meir 1994). In addition, Grossfeld and colleagues showed that the expression of TSP-1 is inversely correlated to p53 expression and to angiogenesis in human bladder cancers (Grossfeld 1997). TSP-1 has also been shown to have both *in vitro* (Bagavandoss 1990, Vogel 1993) and *in vivo* inhibitory effects on angiogenesis (Iruela-Arispe 1997). Interestingly, TSP-1 is also a ligand for $\alpha_v\beta_3$. Activation of $\alpha_v\beta_3$ by TSP-1 has been

Table 3. Anti-angiogenic factors

Endogenous Inhibitors	Activity	Undergone Clincal Trials
Thrombospondin-1 (TSP-1)	Inhibitor of endothelial cell proliferation and migration	No
Platelet factor 4	Inhibitor of endothelial cell proliferation and migration	No
Tissue inhibitors of metalloproteinases (TIMPs) : TIMP-1 TIMP-2 TIMP-3	Inhibitors of both tumor and endothelial cell proliferation and migration	No
Interferon-α	Inhibitor of endothelial cell proliferation and migration, decreases production of bFGF	Yes
Prolactin	Inhibitor of endothelial cell proliferation and migration	No
Tumor-Derived Inhibitors		
Angiostatin	Inhibitor of endothelial cell proliferation, migration and tumor metastases	No
Endostatin	Inhibitor of endothelial cell proliferation and tumor metastases	Yes (Phase I)

shown to disrupt the interactions between endothelial cells and the extracellular matrix, alter cell shape, and result in apoptosis (Dawson 1997).

Angiogstatin, purified from mouse serum and urine, is a 38-kDa inhibitor of angiogenesis (O'Reilly 1994). It represents a specific fragment of the clotting cascade protease precursor plasminogen (Lerch 1980). Angiostatin was shown to inhibit angiogenesis and the growth of metastases by suppressing endothelial cell proliferation and migration. It was initially identified by O'Reilly *et al.* as a circulating molecule produced by primary mouse tumors which can exert inhibitory effect on distant metastases (O'Reilly 1996). Subsequently, it was shown that systemic therapy with angiostatin can inhibit the growth of primary murine tumors and can maintain metastases in a dormant state.

Endostatin, a 20 kDa angiogenesis inhibitor originally purified from a murine hemangioendothelioma, is a fragment of the precursor molecule collagen XVIII (O'Reilly 1997). Endostatin is reported to be a highly active specific inhibitor of endothelial cell proliferation *in vitro*. Systemic treatment with endostatin inhibits angiogenesis, induces tumor dormancy and causes regression of primary tumors (O'Reilly 1997). Moreover, angiostatin and endostatin have the ability to induce regression of tumor blood vessels and complete cessation of tumor growth without the development of drug resistance (Boehm 1997).

Other angiogenesis inhibitors include those which antagonize angiogenic factors such as prostaglandin synthetase inhibitors (Peterson 1986), heparin antagonists which include protamine, platelet factor IV, and the eosinophil major basic protein (Taylor 1982). A variety of steroids (e.g. angiostatic steroids) have also been shown to inhibit

angiogenesis. Angiostatic steroids exert their anti-angiogenic effect partly by reducing plasminogen activator activity, and inhibit endothelial cell migration and proliferation (Pereles 1989, Sakamoto 1988). Some inhibition can be observed in the presence of the steroids alone, but in general the anti-angiogenic activity of the steroid is potentiated by the addition of heparin or related molecules (Sakamoto 1987, Folkman 1989, Rong 1986).

Synthetic inhibitors (Table 4) have also been developed and they were designed to target different stages in the angiogenic process. Among these targets are the vascular growth factors (Rak 1996, Saleh 1996) and receptors (Strawn 1996), cofactors required for the function of angiogenic factors (Wattenberger 1996) and proteases (Min 1996, Brown 1995, Wojtowicz-Praga 1996, Taraboletti 1995) released by migrating endothelial cells. Other synthetic angiogenesis inhibitors include anti-adhesive molecules such as anti-integrin antibodies (Brooks 1994b) and peptide antagonists that block the interaction between integrins and their extracellular matrix ligands (Brooks 1994c).

Some of the better known anti-angiogenesis factors that are in clinical trials are interferon-α (an inhibitor of bFGF production), batimastat (inhibitor of matrix metalloproteinases), thalidomide, and TNP-470. TNP-470 is a synthetic analogue of the fungal antibiotic fumagillin which was demonstrated by Ingber *et al.*. to be a potent angiogenesis inhibitor (Ingber 1990a). It inhibits endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (Ingber 1990a). TNP-470 has been widely used in human trials both as a primary anti-tumor treatment and a sequel to other treatments.

Recently, new strategies based on genetic manipulation of endothelial cells as means of inhibiting angiogenesis have been developed. They are based on the transfer of

Table 4. Novel pharmalogic anti-angiogenic Agents

Agent	Activity	Undergone Clinical Trials
Anti-integrin antibodies	Inhibitors of integrin function, integrin-extracellular matrix interactions (anti-adhesive peptides)	Yes
Anti-integrin peptides		
• **Vitaxin**		Yes (Phase II)
• **EMD121974**		Yes (Phase I/II)
Anti-VEGF antibodies	Inhibitors of angiogenesis activators	Yes (Phase II/III)
SU5416		Yes (Phase I/II)
SU6668		Yes (Phase I/II)
IFN-α		Yes (Phase II/III)
TNP-470	Inhibitors of endothelial cell proliferation, interferes with cell cycle progression	Yes (Phase II)
Thalidomide		Yes (Phase II)
Squalamine		Yes (Phase I)
CA4P		Yes (Phase I/II)
Batimastat	MMP inhibitors, inhibitors of extracellular matrix degradation	Yes
Marimastat		Yes (Phase III)
Bay 12-9566		Yes (Phase III)
Nevostat		Yes (Phase III)
AG3340		Yes (Phase III)
CGS27023A		Yes (Phase I/II)
COL-3		Yes (Phase I)
BMS-275291		Yes (Phase I)

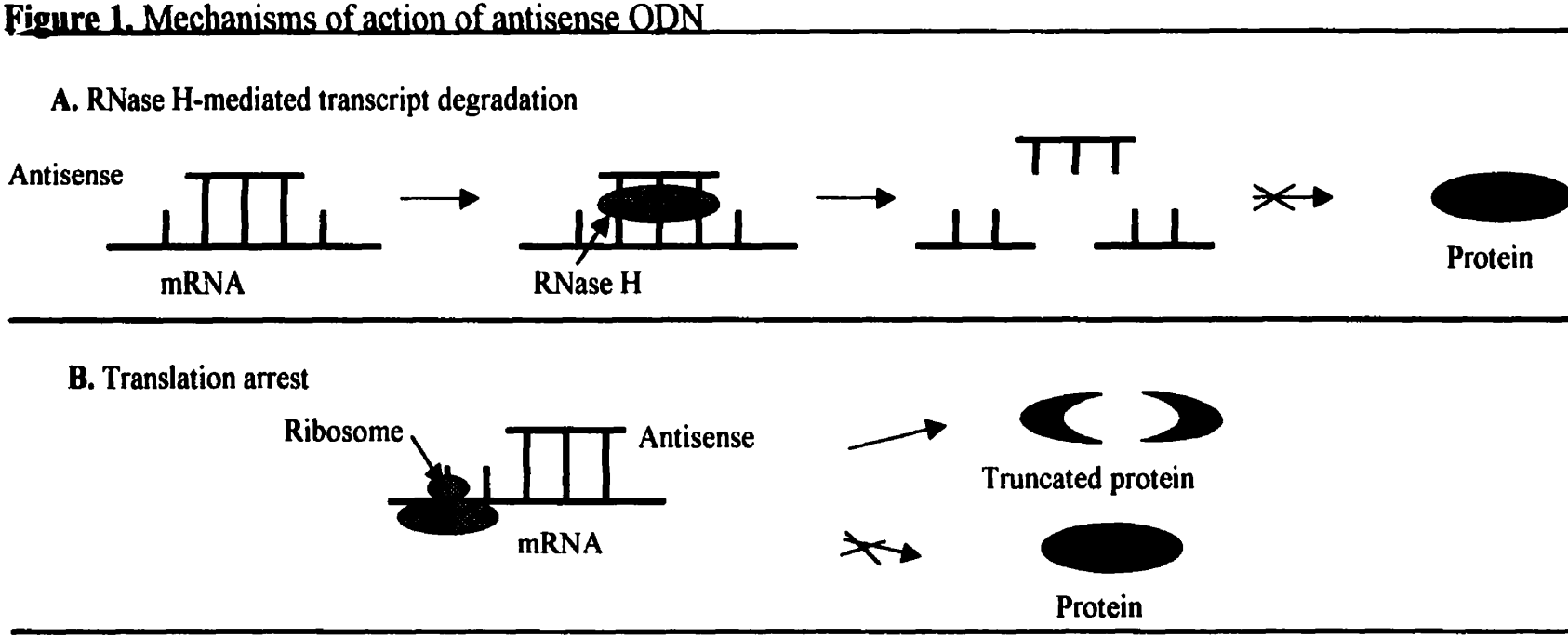
DNA to target cells to produce therapeutic effects. One such group of anti-angiogenesis inhibitors that have shown great promise is the antisense oligodeoxynucleotides (ODN).

1.7 The antisense oligodeoxynucleotdies: potential in therapy

In the late 1960s, the use of antisense ODN was introduced by Gineva and colleagues (Belikova 1967). Antisense ODN are a novel class of therapeutic agents that hold great promise as highly potent drugs. Conceptually, the use of antisense ODN is very attractive, in that all that is required to develop an inhibitor is the sequence of the mRNA of interest and the ability to synthesize the oligonucleotides. The attractiveness of antisense ODN may also be attributed to their ability to suppress selectively the expression and function of proteins involved in diseases, such as cancer. Thus, downregulation of disease-causing genes is one of the most powerful uses of antisense technology. With the increase in gene targets identified by genome sequencing and molecular cloning techniques, the identification of protein functions, along with the improvements in ODN synthesis technology and delivery systems, the antisense field has become increasingly active. The field has now progressed to the point where a number of antisense ODN have been approved for clinical trials.

Inhibition of gene expression by antisense ODN relies on their ability to hybridize to a complementary messenger RNA (mRNA) sequence through Watson-Crick base pairing and consequently prevent translation of the mRNA to its protein product

Figure 1. Mechanisms of action of antisense ODN



(Milligan 1993). Several mechanisms have been proposed for inhibition of protein synthesis by antisense ODN (Helene 1990, Bennett 1994a, Crooke 1995a) (see schematic representation in Figure 1). One of the primary mechanisms is the activation of RNase H (Figure 1A), which cleaves the RNA strand of the RNA-DNA duplex. This mechanism of inhibition was recognized by the direct demonstration of a reduction in the level of target mRNA and the identification of the expected cleavage products (Stewart 1996, Condon 1996, Giles 1995). There are also steric mechanisms by which antisense ODN can inhibit protein expression. Translation arrest (Figure 1B), in which the ODN hybridizes to the target mRNA and blocks movement of the ribosome and consequently mRNA translation, is one of the most cited steric effects (Lengyel 1961). Another related mechanism is prevention of ribosome assembly on the mRNA strand (Baker 1997). Inhibition of RNA processing (Figure 1C) by the prevention of intron splicing is also a potential mechanism of action by which antisense ODN may inhibit or alter gene expression (Sierakowska 1996, Hodges 1995). Disruption of the secondary or tertiary structures of RNA such as stem-loop complexes (Figure 1C) can weaken mRNA stability and cause its degradation. Other mechanisms by which antisense ODN may inhibit mRNA processing include the inhibition of 5' capping or 3'polyadenylation (Figure 1C), both of which destabilize the mRNA. In sum, there are multiple mechanisms by which antisense oligonucleotides can suppress gene expression. The mechanism of action is dependent in part on where on the mRNA the oligonucleotide hybridizes as well as on the specific oligonucleotide chemistry used.

To overcome the stability problems caused by the activity of exo- and endonucleases and to increase the half-life of the antisense ODN, chemical modifications

have been made to the phosphate backbone, the deoxyribose moiety and the bases of the oligonucleotide structures to produce more stable oligonucleotides (Manoharan 1992). Of the many chemical modifications reported to date, the most widely used include substitution of one of the non-bridging oxygen atoms on the phosphate group with either a methyl group or a sulfur atom, to produce methyl-phosphonate (Agris 1986) and phosphorothioates (Eckstein 1989, Matsukura 1987), respectively. Apart from protecting the oligonucleotide from degradation by nucleases, phosphorothioate oligonucleotides also support RNase H-mediated hydrolysis of the target mRNA (Akhtar 1991, Monia 1993). The stability of phosphorothioate oligonucleotides varies depending on the cell line investigated. In cell-based assays, phosphorothioate oligonucleotides have been reported to reduce expression of the targeted mRNA for 24-48 hours (McKay 1996, Dean 1994, Monia 1996a). If longer suppression of target mRNA is desired, then cells can be treated repeatedly with the oligonucleotides (Altman 1996). Several lines of evidence indicate that the optimal length for phosphorothioate oligonucleotides ranges from 17-25 bases (Wagner 1996), as longer ODN have the potential to partially hybridize with non-target mRNA sequences (Lima 1992).

One potential problem associated with the use of antisense ODN is non-specific side effects (Milligan 1993, Stein 1993, Helene 1990). These non-specific biological effects of ODN occur when they bind with DNA sequences, proteins and small molecules other than their specific target. When an antisense molecule causes a biological effect, it is extremely difficult to determine whether the effect occurred because the ODN interacted specifically with its target mRNA or due to some non-antisense reaction involving other nucleic acids or proteins (Stein 1995, Branch 1996). Therefore, for all

antisense studies, it is imperative to include the proper controls in order to demonstrate specific gene inhibition. An antisense molecule is typically considered to be specific if two criteria are met. First, there is no gross loss of cell viability. Secondly, the levels of the target mRNA and its transcriptional product decrease significantly more than those of control mRNAs.

No studies demonstrating the effects of α_v antisense phosphorothioate oligonucleotides on angiogenesis have yet been reported. However, numerous studies have been published claiming inhibition of a wide variety of other gene products with the application of antisense phosphorothioate oligonucleotides in mammalian cell culture-based experiments (Milligan 1993, Stein 1993, Helene 1990) and the use of antisense strategies to treat various cancers in animal models has also been very productive. A phosphorothioate ODN targeted to *c-myb* inhibited human leukaemia-cell growth in SCID mice and increased survival time by 3.5-fold (Ratajaczak 1992, Hijya 1994). Similar encouraging results were obtained with a phosphorothioate ODN targeted to the *bcl-2* gene where there was inhibition of *bcl-2* expression and the oncogenic potential of B-cell lymphoma cells in SCID mice (Cotter 1994). Finally, intravenous administration of a phosphorothioate ODN targeted to *c-raf* kinase resulted in a dose-dependent reduction of tumor growth in nude mice implanted with cultured human tumor cells (Monia 1996b). In addition, Miele *et al.* have utilized antisense oligonucleotides to downregulate expression of endothelial cell adhesion molecules as means of treating cancers. For instance, an intraperitoneal administration of an antisense ODN against intercellular adhesion molecule 1 (ICAM-1) has been demonstrated to reduce melanoma metastasis in mice (Miele 1994).

Based on the promising cell-culture and animal studies, several antisense oligonucleotides have recently been approved for clinical trials. Clinical evaluation of GEM 91, a 25-mer antisense phosphorothioate ODN which binds to the *gag* region of HIV RNA has recently been initiated (Lisziewicz 1994). Formivirsen is a phosphorothiate ODN currently in use in Phase III trials as a locally administered treatment of CMV retinitis (Kisner 1997). Finally, ISIS 2302, an anti-inflammatory inhibitor of ICAM-1 gene expression, is currently in Phase II trials as a treatment for Crohn's disease (Yacyshyn 1998), ulcerative colitis, and rheumatoid arthritis.

1.8 Rationale for the present study

Quiescent endothelial cells lining the blood vessels rest on a basement membrane. During the course of angiogenesis, endothelial cells penetrate this basement membrane and continue to migrate and proliferate in the perivascular tissue stroma. The ability to penetrate the vascular basement membrane is an aspect of endothelial cell behaviour unique to angiogenesis. The degradation of basement membrane macromolecules is achieved by proteases which have been shown to be produced at elevated levels by malignant cells such as fibrosarcoma cells (Loskutoff 1977, Salo 1982, Laug 1980). One of the most important proteases in this context is urokinase plasminogen activator (uPA) which is active when bound to its membrane-linked receptor (uPAR). uPA is a serine protease which converts serum plasminogen to plasmin which in turn can degrade a

broad spectrum of extracellular matrix proteins, including fibronectin (Balian 1979) and laminin (Liotta 1981). Plasmin can also activate collagenase, which is capable of degrading types I, II and III collagens (Werb 1977, Paranjpe 1980). Endothelial cell production of uPA and uPAR were shown to rise in response to angiogenic factors (Gross 1982, 1983, Kalebic 1983).

Previous experiments in Dr. Brodt's laboratory have shown that α_v antisense phosphorothioate oligonucleotides suppress not only α_v but also uPAR levels in human metastatic melanoma cells (Nip 1995). Based on these findings and the recent studies demonstrating the importance of α_v in angiogenesis, we investigated in the present study the effect of α_v antisense ODN on expression of α_v in human endothelial cells and its effect on endothelial cell migration and proliferation. We rationalized that if α_v antisense phosphorothioate oligonucleotides suppress α_v synthesis in endothelial cells and reduce endothelial cell migration and proliferation, they could serve as inhibitors of angiogenesis, since these three parameters are critical to the process of angiogenesis. Successful "cell adhesion-targeted therapy" could represent a major new approach for the prevention of human diseases involving abnormal angiogenesis such as tumor growth and metastasis.

Chapter 2

Materials and Methods

2.1 Human umbilical vein endothelial cell (HUVEC) cultures

Umbilical cords were obtained after normal delivery from the Surgical Pathology Laboratory of the Royal Victoria Hospital (Montreal, Quebec, Canada). They were stored at 4°C in a saline solution containing 238.3 mg/ml N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Gibco, Burlington, Ontario, Canada), 2 mg/ml anhydrous D-glucose (Sigma, Oakville, Ontario, Canada), and 0.3 mg/ml KCl (Fisher, Nepean, Ontario, Canada). Umbilical cord veins were cannulated and flushed with Ringer's solution to remove all traces of blood and thrombus, and then the luminal surfaces of the veins were filled with 0.1% collagenase (Sigma) in phosphate-buffered saline (PBS) (Gibco) and incubated for 15 min at 37°C to detach endothelial cells. The cells were collected by flushing the veins with Medium-199 (Gibco) and centrifugated for 7 min at 1000 rpm. Pellets were resuspended in Medium-199 (basic medium) supplemented with 20% fetal calf serum (FCS) (Wisent, St. Bruno, Quebec, Canada), 238.3 mg/ml HEPES (Gibco), 10,000 U/ml penicillin, 10,000 µg/ml streptomycin, 29.2 mg/ml L-glutamine (all purchased from Gibco), and with 1.5 mg/ml endothelial cell growth supplement (ECGS), and 1500 U/ml heparin (both obtained from Sigma) (HUVEC medium). Cells isolated in this manner were previously identified as >90% endothelial in origin as confirmed by morphology (cobblestone-like) and positive immunofluorescence staining with an antibody to von Willebrand factor (Hoyer 1973, McGill 1998). Isolated endothelial cells were plated into 75-cm^2 tissue culture flasks (Sarstedt, St. Leonard, Quebec, Canada) which were pre-coated with 0.3% gelatin (Fisher) to improve cell attachment, and incubated at 37°C in a 5% CO_2 incubator. The next day, cells were gently washed twice

with Medium-199 and fresh HUVEC medium was added. New culture medium was replenished on alternate days. HUVEC cultures of 80-90% confluency were used between the first and fifth passages.

2.2 Reagents

Oligonucleotides. For the first stage of the study, the effect of the following three α_v antisense phosphorothioate oligonucleotide sequences on α_v expression by HUVEC was analyzed.

1. AS 1: 5'-TCAGCATCAATATCTTGT-3', complementary to bases 563 - 580 of the human α_v subunit sequence
2. AS 2: 5'-AAGCCATCGCCGAAGTGC- 3', complementary to bases 31 - 48 of the human α_v subunit sequence
3. AS 3: 5'-GACTGTCCACGTCTAGGT-3', complementary to bases 136 - 153 of the human α_v subunit sequence

Control oligonucleotide (Sense 2): 5'-GCACTTCGGCGATGGCTT-3', an eighteen-base sense sequence corresponding to AS 2

Initially, these oligonucleotides were used in Dr. P. Brodt's laboratory (Department of Surgical Research, Royal Victoria Hospital, Montreal, Quebec, Canada) to determine their effect on α_v and uPAR expression in human melanoma cells. The oligonucleotides were synthesized by Sheldon Biotechnology Center (Montreal, Quebec, Canada) and purified three times using ethanol precipitation (2.5 vol of ethanol and 1/4

vol of 10M ammonium acetate). Subsequently, the following oligonucleotides were obtained as a kind gift from ISIS Pharmaceuticals (Carlsbad, CA, USA).

1. ISIS 15630 (AS 2): described above

As controls,

2. ISIS 16205: 5'-CAAGGTCGCACACCACCTGC-3', a sequence complementary to the mouse α_v sequence with no homology to the human α_v sequence

Antibodies. Rat MAb 69-6-5 to human integrin subunit α_v (Lehmann 1994) was a kind gift from Dr. J. Marvaldi (Laboratoire de Biochimie Cellulaire, Universite d'Aix-Marseille, Marseille, France). A peroxidase-conjugated goat anti-rat immunoglobulin was used as a secondary antibody. It was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

2.3 Cell treatment with oligonucleotides

HUVEC monolayers of 80-90% confluency were dispersed with 0.05% trypsin-EDTA (Sigma) and plated in 0.3% gelatin-coated 25-cm^2 tissue culture flasks (Sarstedt). The cells were allowed to spread in HUVEC medium. After 4-5 h, oligonucleotides were added at the desired concentrations and this was repeated 24 h later for a total incubation time of 2 days at which time the oligonucleotides were removed and fresh HUVEC medium was added.

2.4 Quantitation of cell surface α_v expression

Expression of cell surface α_v expression was assessed by the Enzyme-linked immunosorbent assay (ELISA). Cells were treated with oligonucleotides for 48 h in 0.3% gelatin-coated 96-well tissue culture plates (Sarstedt) in HUVEC medium. The cells were washed five times with PBS and non-specific protein binding sites were blocked with 1% bovine serum albumin (BSA) (Boehringer Mannheim, Laval, Quebec, Canada) in PBS for 1 h at room temperature. The cells were fixed with 0.125% glutaraldehyde (Fisher) in PBS for 2 min and rinsed five times with PBS containing 0.1% BSA (assay buffer). To each well, 10 μg/ml MAb 69-6-5 diluted in assay buffer was added for a 90-min incubation at room temperature. Unbound antibody was removed by washing the cells five times with assay buffer. A peroxidase-conjugated goat anti-mouse IgG diluted 1:1000 in assay buffer was used as a secondary antibody and incubated with the cells for 60 min at room temperature. Unbound antibody was removed by 5 washings with assay buffer. A colorimetric reaction was initiated with ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (Boehringer Mannheim) as a substrate and stopped 8-10 min later by the addition of 0.05% sodium azide (Fisher). Color intensity was measured with a ThermoMAX Microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA) at a wavelength of 405 nm. The program used to analyze the data was SOFTmax 2.32 Software package for the MAXline Microplate Readers (Molecular Devices Corporation).

2.5 Endothelial cell migration assay

Cell migration was measured using 8.0-μm nucleopore filters (Fisher) pre-coated with 0.3% gelatin. The filters were placed into 24-well tissue culture plates (Sarstedt) and $4x10^4$ cells in Medium-199 containing 0.1% BSA were evenly loaded onto each filter. VEGF at a concentration of 25 ng/ml or bFGF at a concentration of 10 ng/ml (both purchased from Intergen Company, Purchase, NY, USA) with or without l0 μg/ml human fibronectin (Roche, Burlington, Ontario, Canada) diluted in Medium-199 supplemented with 0.1% BSA were placed in the lower chamber to induce cell motility. Cell migration was measured 8-24 h later. Cells were fixed with 0.125% glutaraldehyde for 20 min and stained with 0.5% crystal violet (Fisher). All non-migrating cells were removed from the upper face of the filter with a cotton swab. Migrating cells on the lower surface of the filter were enumerated using a Nikon Diaphot-TMD inverted microscope (Nikon Canada, Montreal, Quebec, Canada) equipped with an ocular square millimeter grid. To assess the effects of α_v antisense ODN on endothelial cell migration, HUVEC were treated with 5-40 μM ODN for 48 h at 37^0C. Cells were dispersed and loaded onto gelatin-coated nucleopore filters at a density of 4×10^4 cells/filter in Medium-199 containing 0.1% BSA. Cell migration was measured after a 24 h incubation at 37^0C. To induce cell motility, 20 ng/ml bFGF or 25 ng/ml VEGF in 0.1% BSA were added to the lower chambers together with 10 μg/ml fibronectin.

2.6 Endothelial cell proliferation assay

HUVEC were treated with the oligonucleotides first in complete HUVEC medium and 24 h later, in Medium-199 supplemented with 1% FCS. On the following day, the cells were dispersed with 0.05% trypsin-EDTA and seeded at a density of $5x10^3$ cells per well in 96-well tissue culture plates pre-coated with 0.3% gelatin. The cells were incubated for 48-72 h at 37°C in Medium-199 containing 1% FCS and bFGF at a concentration of 20 ng/ml. The cells were pulsed for 18 h with 1.0 μCi/ml [^{3}H]-thymidine (50-90 mCi/mmol, Mandel Scientific Company Ltd., Guelph, Ontario, Canada), lysed by repeated freezing and thawing, harvested using the Skatron Cell Harvester (Skatron Instruments Inc., Sunnyvale, CA, USA) and absorbed onto Filtermats filter papers (Skatron Instruments Inc.). The Filtermats were added into scintillation tubes (Skatron Instruments Inc.) containing 3 ml of CytoScint scintillation cocktail (ICN, Costa Mesa, CA, USA) and radioactivity was measured in a LKB 1217 Rackbeta liquid scintillation counter (LKB, Helsinki, Finland). Cells incubated in HUVEC medium or in Medium-199 containing 1% FCS served as controls for maximal or baseline uptake levels, respectively.

2.7 MTT assay

To determine cell viability after treatment with the oligonucleotides, the cells were seeded onto 0.3% gelatin-coated 96-well tissue culture plates (Sarstedt) at a density of $5x10^3$ cells per well in 200 μl medium containing 5-40 μM antisense ODN. At the

intervals indicated in the text, 10 µl of a 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) solution were added to each well and the plates were incubated for 4 h at 37°C. To each well, 160 µl of dimethyl sulfoxide (DMSO) (Fisher) were added and the wells mixed thoroughly to dissolve the dark blue formazan crystals formed. Color intensity was measured with a ThermoMax Microplate reader (Molecular Devices Corporation) at a wavelength of 550 nm. The program used to analyze the data was SOFTmax 2.32 Software package for the MAXline Microplate Readers (Molecular Devices Corporation).

2.8 Statistics

Student's *t* test was used to analyze migration and proliferation data.

Chapter 3

Results

3.1 Identification of an α_v antisense oligonucleotide with a potent inhibitory effect on cell surface α_v gene expression

Three antisense oligonucleotides sequences previously shown to suppress α_v expression in human melanoma cells (see "Materials and Methods") were tested for their effect on α_v expression in an early passage HUVEC culture. Results shown in Fig. 1 demonstrate that AS 2 had the most potent inhibitory activity on cell surface α_v levels. At a concentration of 40 μM, the level of α_v decreased by 78%. Neither of the other two ODN, i.e. AS 1 and AS 3, reduced α_v expression. AS 2 was therefore selected for all subsequent studies.

The inhibitory effect of AS 2 on α_v expression was confirmed with a second HUVEC culture (Fig.2). With these cells, the inhibition was shown to be dose-responsive and reached a maximum of 50% at a concentration of 40 μM ODN. A sense ODN control showed no effect.

In addition, antisense-treated cells lost adhesiveness and became rounded in morphology thereby confirming the reduction in α_v expression (Fig. 3). This loss of adhesiveness of antisense ODN-pretreated endothelial cells was further confirmed by the yield of cells from harvesting after ODN treatment relative to untreated cells (data not shown).

3.2 HUVEC migration in response to VEGF and bFGF

HUVEC migration in respnse to VEGF and bFGF was assessed using different experimental conditions. Cells were placed onto gelatin-coated nucleopore filters and the filters placed in wells of 24-well plates for 8 or 24 h at 37^0C. Results (Fig. 4) showed that the relative increase in HUVEC migration was optimal in the presence of 20 ng/ml bFGF or 25 ng/ml VEGF, when these factors were combined with the extracellular protein fibronectin (10 μg/ml). Under these conditions, migration at 8 and 24 h increased up to 24 (bFGF)- and 29 (VEGF)-fold relative to unstimulated cells (control). Growth factors alone (i.e. VEGF, bFGF) failed to induce stimulation whereas fibronectin alone had a minor effect, that is 12% of maximal stimulation (data not shown).

3.3 Induction of cell proliferation by bFGF

To determine optimal conditions for HUVEC proliferation, the cells were grown in Medium-199 containing 0.5% or 1% serum. Twenty four hours later, the cells were stimulated with 10 or 20 ng/ml bFGF for 48 h. Results in Fig. 5A show that cell numbers increased under all conditions used. Maximal increase (5-fold) was seen when the cells were incubated for 48 h in the presence of 20 ng/ml bFGF and 1% serum. Results in Fig. 5B show an increase of 10-fold in ^{3}H-thymidine uptake under these experimental conditions (i.e. the 48 h used in MTT). Maximal increase (21-fold) was seen when cells were stimulated with 20ng/ml bFGF for 72 h. Cells incubated in medium containing 1% FCS in the absence or presence of bFGF are shown in Fig. 6A and 6B respectively. The

difference in morphology of the cells treated in media containing 1% FCS as compared to cells treated with HUVEC media containing 20% FCS (Fig. 1) was due to the different concentration of serum in the media. Serum plays an important role determining the morphology of cells; in its absence or at a low concentration, cells tend to become rounded in morphology because they don't adhere and spread as well.

3.4 Variability in the response to α_v antisense ODN between endothelial cell cultures

We examined the effect of AS 2 on α_v expression in multiple cultures derived from individual cords, simultaneously. Results shown in Fig. 7 demonstrate that the effect of ODN treatment differed among different cell cultures and ranged from no reduction to an 80% reduction in the level of α_v expression in cells treated with AS 2. A murine α_v antisense ODN showed no effect.

3.5 Inhibition of cell migration by α_v antisense oligonucleotides

The effect of α_v antisense ODN on cell migration was analyzed. HUVEC were treated with 5-40 μM ODN for 48 h, then dispersed and placed onto gelatin-coated nucleopore filters for a 24 h incubation at 37^0C in the presence of VEGF and fibronectin. Results shown in Fig. 8 demonstrate that the inhibition of HUVEC migration was dose-dependent and could be reduced by up to 95 % at 40 μM, relative to untreated controls. The results shown in Fig. 10 demonstrate that cells derived from different umbilical cords vary in the magnitude of their response to migration-inducing factors as well as in their

sensitivity to the inhibitory effects of both human (AS 2) and murine (Mouse ODN) α_v antisense ODN. The difference in the reduction caused by AS 2 and Mouse ODN was significant for endothelial cell cultures A ($p<0.0005$), B ($p<0.005$), and C ($p<0.05$).

3.6 Inhibition of cell proliferation by α_v antisense oligonucleotide

The effect of α_v antisense ODN on cell proliferation was next examined. HUVEC cells were pretreated with 20 or 40 μM ODN for a total of 48 h and transferred to gelatin-coated 96-well plates for the ^{3}H-thymidine uptake assay performed as described previously (see Materials and Methods). Results shown in Fig. 11 demonstrate that, at a concentration of 40 μM, AS 2 could reduce cell proliferation by up to 69% and 78% following 48 and 72 h incubations respectively. We found that the murine α_v specific ODN used at the same concentration, also inhibited cell proliferation but at a significantly lower magnitude ($p<0.05$).

3.7 α_v antisense oligonucleotides were not cytotoxic to HUVEC

To confirm that the inhibitory effect of the ODN was not due to non-specific cytotoxic effects, cell survival was assessed after ODN treatment using the MTT assay. Following treatment with 5-40 μM ODN, the cells were plated into gelatin-coated 96-well plates and cell survival was analyzed under several culture conditions using different serum concentrations. As shown in Fig. 12A, in the presence of medium containing 1% FCS, after treatment with AS 2, no significant cell death was observed relative to

untreated cells under identical conditions. Since AS 2 is an antisense targeted at α_v which plays a critical role in promoting cell adherence and spreading, its addition causes cells to become rounded in morphology and eventually be lifted off and remain in suspension. The MTT assay described in Fig. 12 was done without the washing of cells and the removal of supernatant; therefore, the number of surviving cells observed in Fig. 12 differs significantly from the number of cells seen in Fig. 3A.

Figure 1. Identification of an α_v antisense oligonucleotide with a potent inhibitory effect on α_v gene expression

HUVEC cells were treated with α_v antisense phosphorothioate oligonucleotides at a concentration of 40 μM for 48 h at 37^0C. $\alpha_v\beta_3$ expression was measured by ELISA. Cells were washed with PBS and fixed with 0.125% glutaraldehyde. To each well, 50 μl of 0.1% BSA (negative control) or MAb 69-6-5 (10 μg/ml) were added for a 90 min incubation followed by a 60 min incubation with a peroxidase-conjugated goat anti-mouse antibody (1:1000). ABTS was used as a substrate and the color intensity was measured at 405 nm. Results are expressed as percent expression relative to untreated cells and based on experiments done in triplicates. AS 2 was selected for further studies.

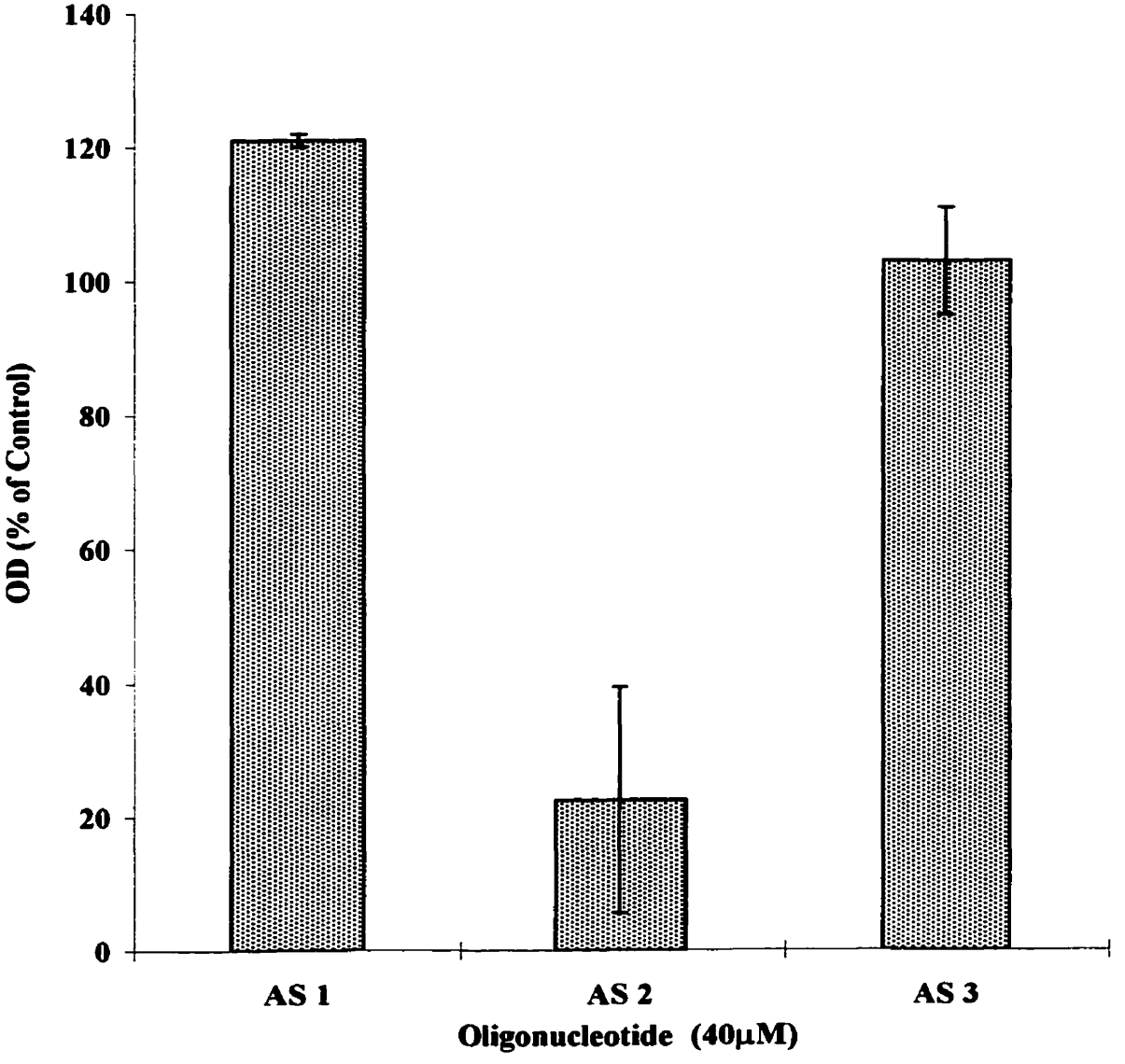

140
120
100
80
60
40
20
0
OD (% of Control)
AS 1
AS 2
AS 3
Oligonucleotide (40μM)

Figure 2. Inhibition of α_v expression by AS 2 was dose dependent

HUVEC were treated with AS 2 at concentrations of 5-40 μM for 48 h at 37^0C. $\alpha_v\beta_3$ expression was measured by ELISA as described in the legend to Fig. 1. Results are expressed as percent expression relative to untreated cells. A phosphorothioate 18-base sense sequence corresponding to AS 2 (Sense 2) was used as the control ODN.

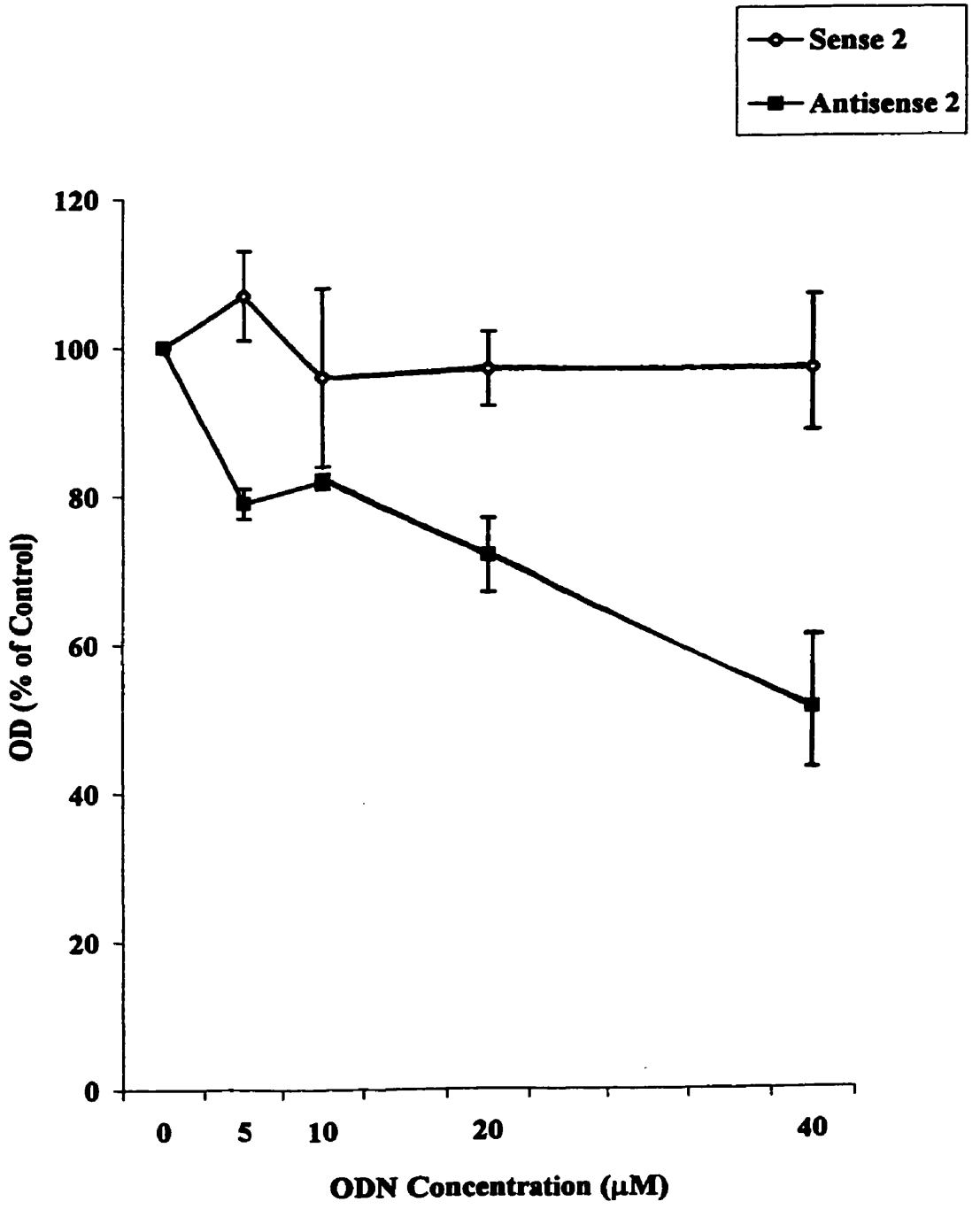
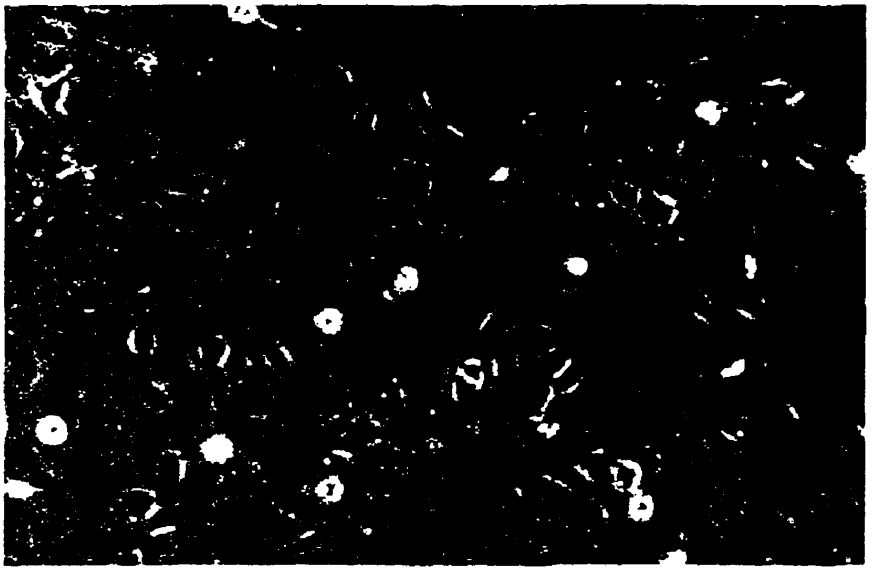

Sense 2
Antisense 2
120
100
80
60
40
20
0
OD (% of Control)
0
5
10
20
40
ODN Concentration (μM)

Figure 3. Light microscopy view of HUVEC pre-treated with antisense oligonucleotides

Assay conditions were those described in the legend to Fig. 1. Cells were treated with AS 2 for 48 h. Shown are cells untreated with (A) or treated (B) with AS 2 (Mag:x500).

A



B

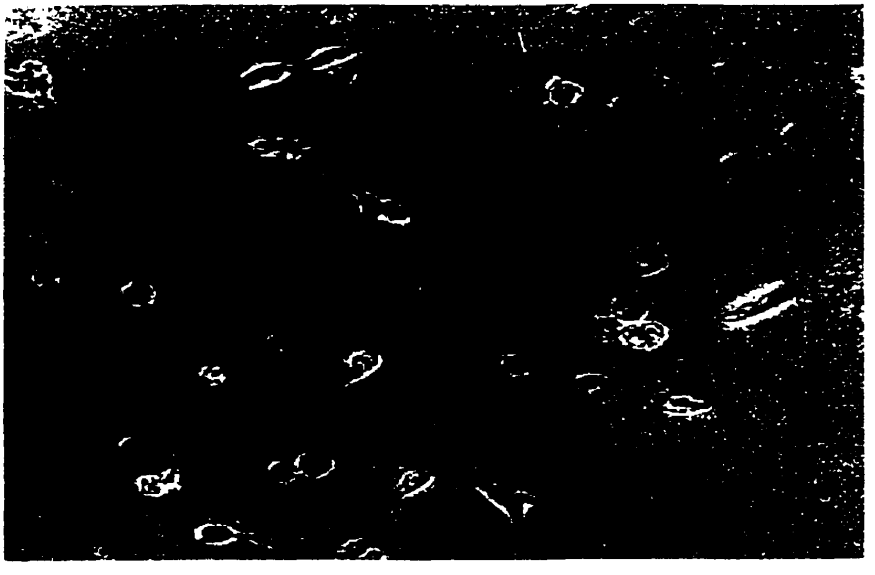
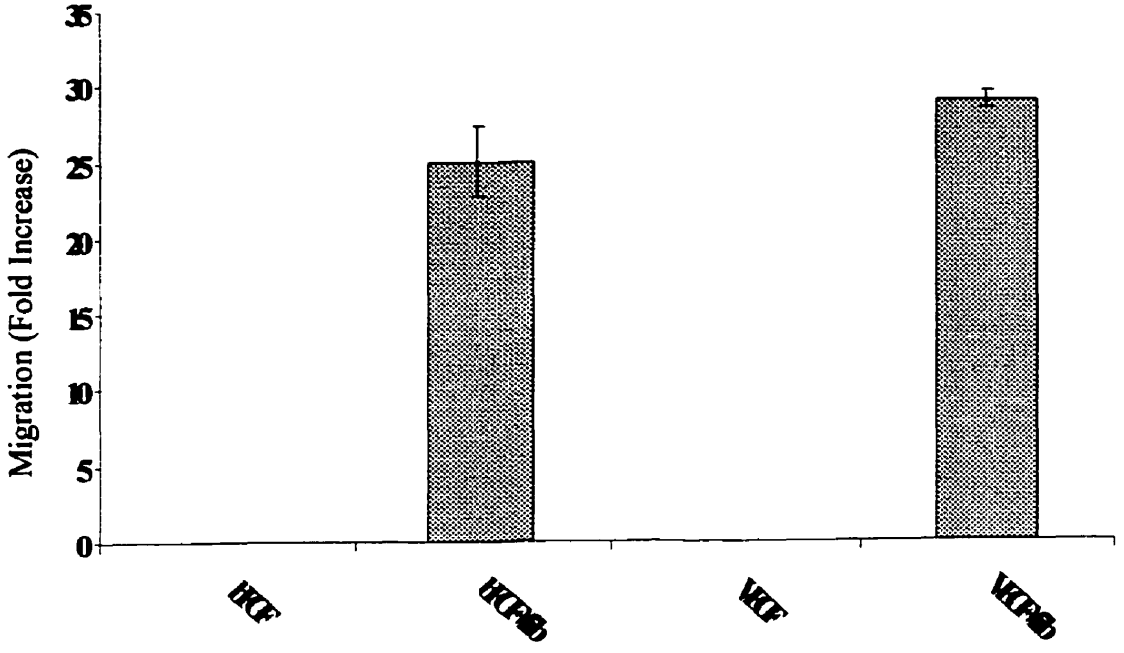


Figure 4. Induction of HUVEC migration by VEGF and bFGF

Cell migration was measured at 8 or 24 h incubation at 37^0C. HUVEC were seeded onto gelatin-coated nucleopore filters at a density of 4 x 10^4 cells/filter in Medium-199 containing 0.1% BSA. To induce cell motility, 20 ng/ml bFGF or 25 ng/ml VEGF, in 0.1% BSA were added to the lower chambers together with 10 μg/ml fibronectin. Results are expressed as fold increase in migration relative to cells incubated in the absence of migration factors. All experiments were performed in duplicate. At 8 h, the mean number of cells which migrated in control wells in the absence of chemoattractants was 8 ± 2.8. At 24 h, the mean number of migrating cells in the control wells 28 ± 7.8.

A


Migration (Fold Increase)
35
30
25
20
15
10
5
0
HCF
HCF+bFGF
VCF
VCF+bFGF

B

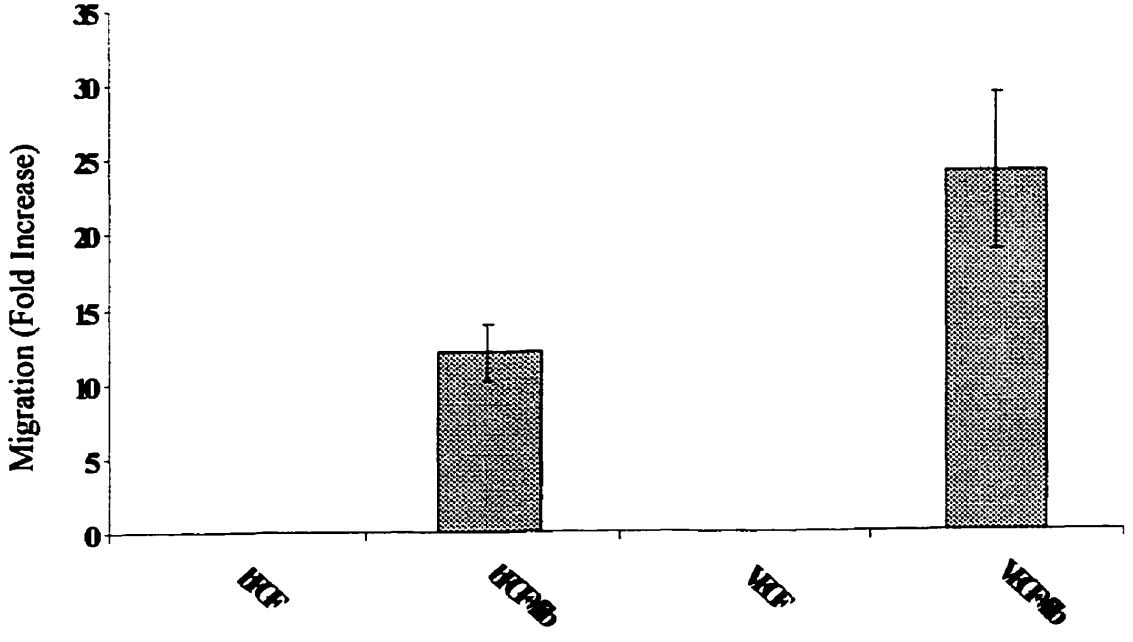
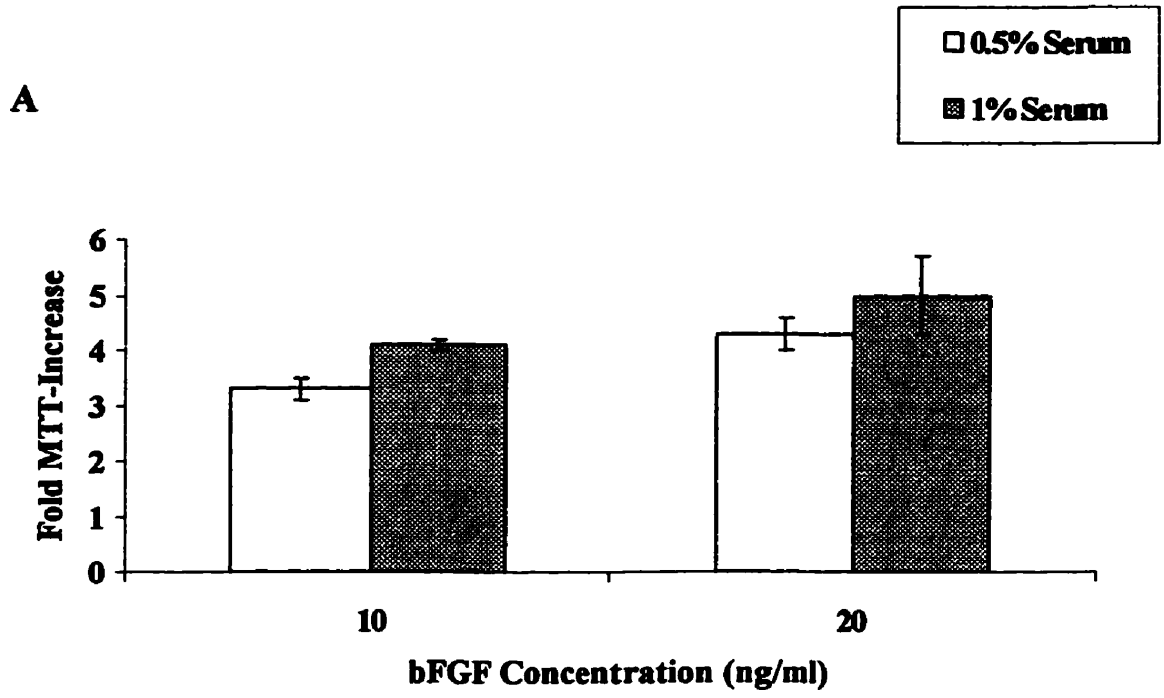

Migration (Fold Increase)
35
30
25
20
15
10
5
0
HCF
HCF+bFGF
VCF
VCF+bFGF

Figure 5. Stimulation of cell proliferation in response to bFGF

A. HUVEC were grown in Medium-199 supplemented with 0.5% or 1% FCS for 24 hours. The cells were then plated into gelatin-coated 96-well plates and stimulated with 10 or 20 ng/ml bFGF for 48 hours. Induction of cell proliferation was measured by the colorimetric MTT assay. Color intensity was measured at 550 nm. Results are expressed as fold increase in absorbance relative to cells cultured in the absence of bFGF.

B. HUVEC were grown in Medium-199 supplemented with 1% FCS for 24 h. Cells were seeded into gelatin-coated 96-well plates with different concentrations of bFGF diluted in Medium-199 containing 1% FCS and incubated for 24-72 h at 37^0C. ^{3}H-thymidine incorporation was measured after pulsing for 18 h. Results are expressed as fold stimulation relative to cells cultured in the absence of bFGF and based on two experiments done in triplicate.

A


0.5% Serum
1% Serum
Fold MTT-Increase
0
1
2
3
4
5
6
10
20
bFGF Concentration (ng/ml)

B

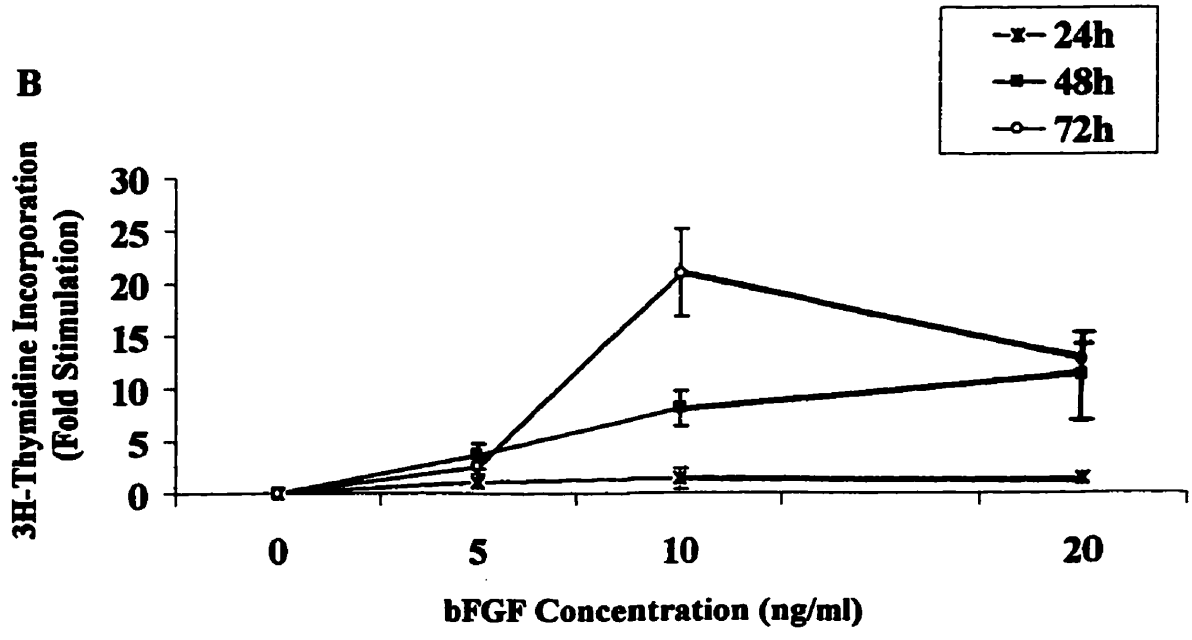

24h
48h
72h
3H-Thymidine Incorporation (Fold Stimulation)
0
5
10
15
20
25
30
0
5
10
20
bFGF Concentration (ng/ml)

Figure 6. Light microscopy view of HUVEC proliferation in response to bFGF

Assay conditions were those described in the legend to Fig. 5. Briefly, HUVEC were grown in Medium-199 supplemented with 1% FCS for 24 hours. The cells were then plated into gelatin-coated 96-well plates and incubated for 48h in medium containing 1% FCS and 20 ng/ml bFGF. Shown are cells incubated in the absence (A) or presence (B) of bFGF (Mag: x500).

A

B

Figure 7. Variability in the response to α_v antisense ODN between endothelial cell cultures

HUVEC were treated with 40 μM ODN for 48 h and seeded into gelatin-coated 96-well plates at a density of 5 X 10^3 cells/well. $\alpha_v\beta_3$ expression was determined 24 h later with ELISA as described in the legend to Fig. 1. Mouse α_v antisense ODN was used as a control. Results are expressed as percent expression relative to untreated cells. Endothelial cell cultures A-E were derived from five individual cords.

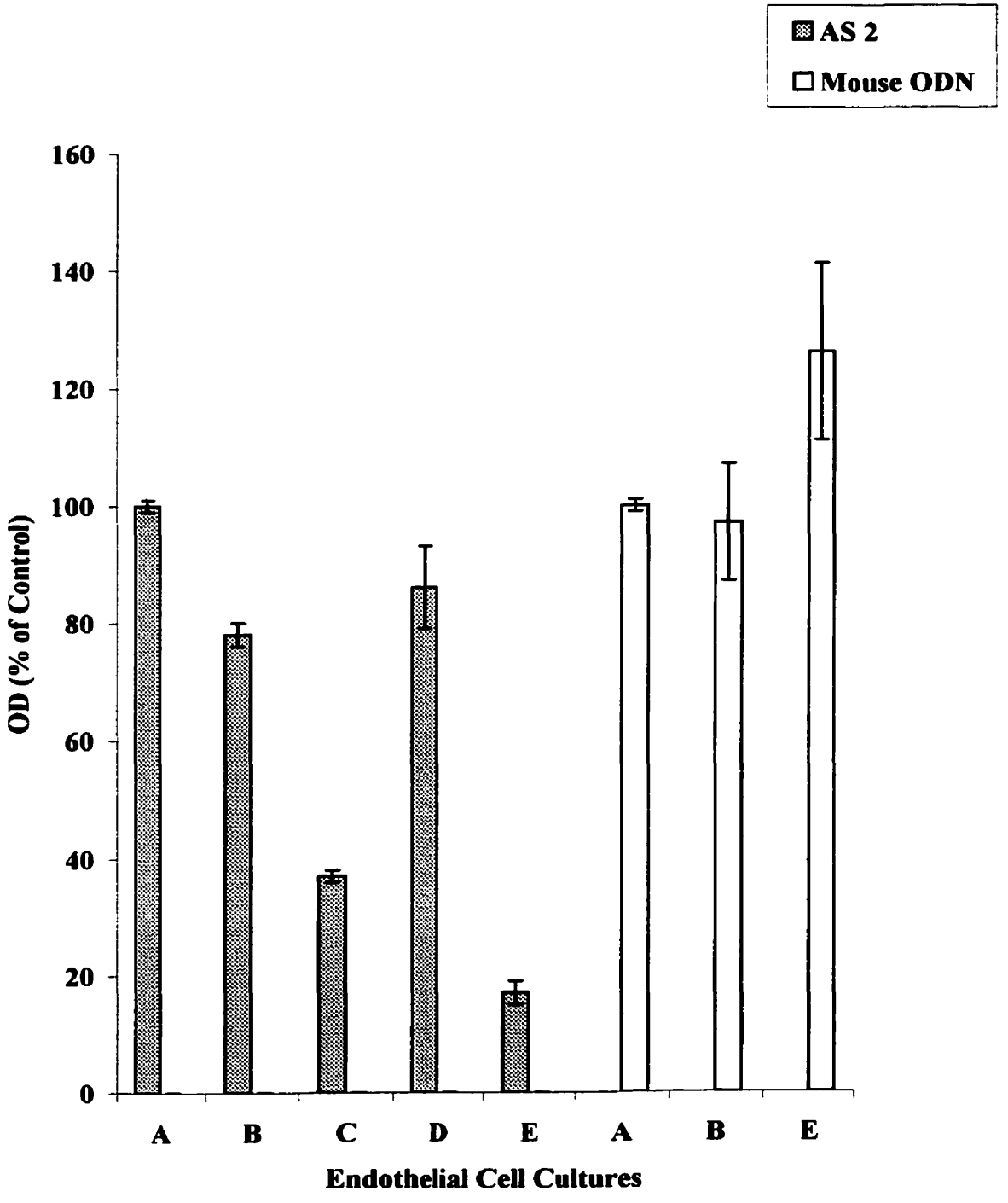

AS 2
Mouse ODN
160
140
120
100
80
60
40
20
0
OD (% of Control)
A
B
C
D
E
A
B
E
Endothelial Cell Cultures

Figure 8. Inhibition of cell migration by α_v antisense oligonucleotides was dose dependent

HUVEC were treated with 5-40 μM ODN for 48 h at 37^0C. Cells were dispersed and loaded onto gelatin-coated nucleopore filters for a 24 h incubation at 37^0C in the presence of VEGF and fibronectin. Results are expressed as fold increase in migration relative to cells incubated in the absence of VEGF and fibronectin.

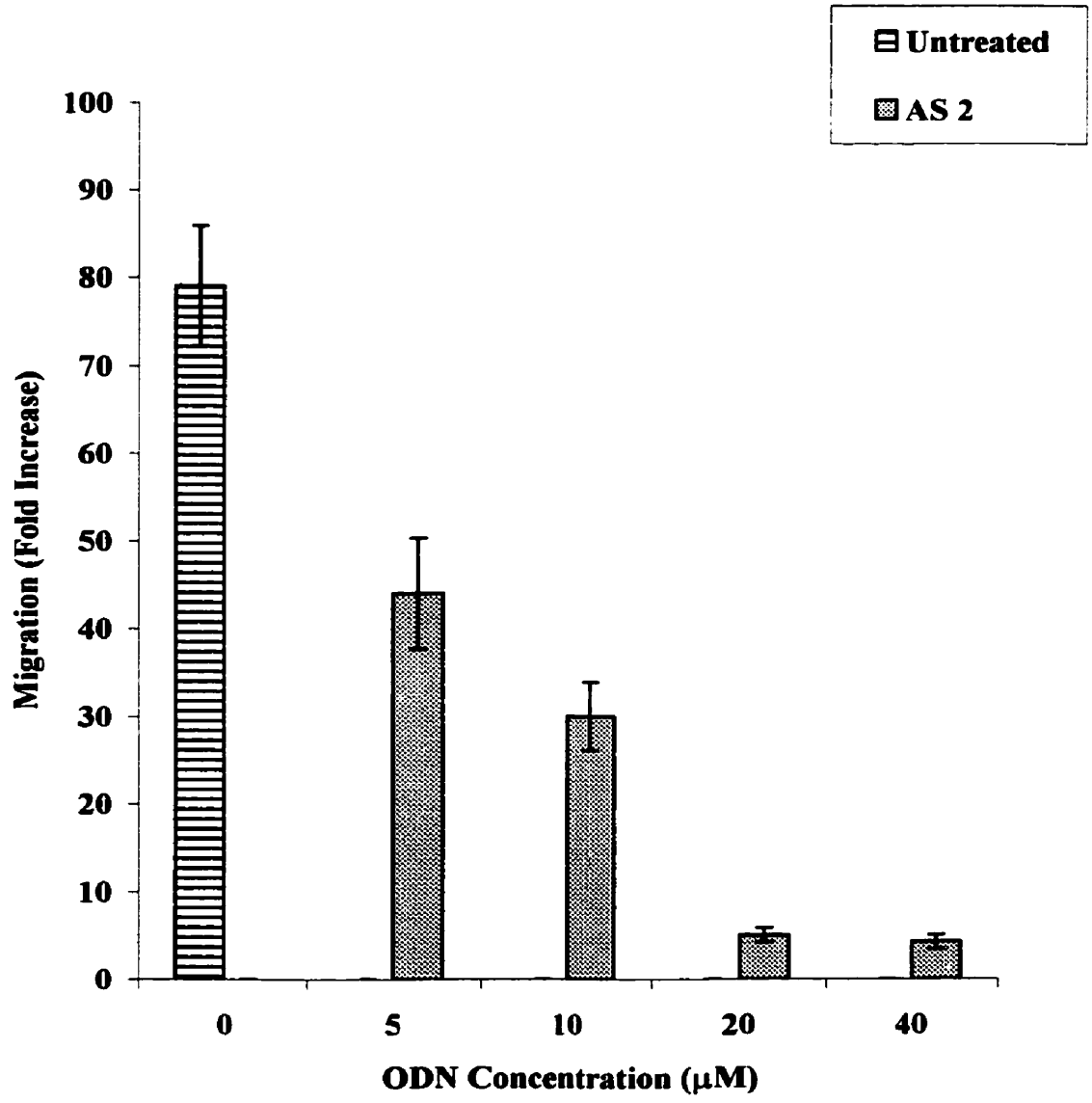
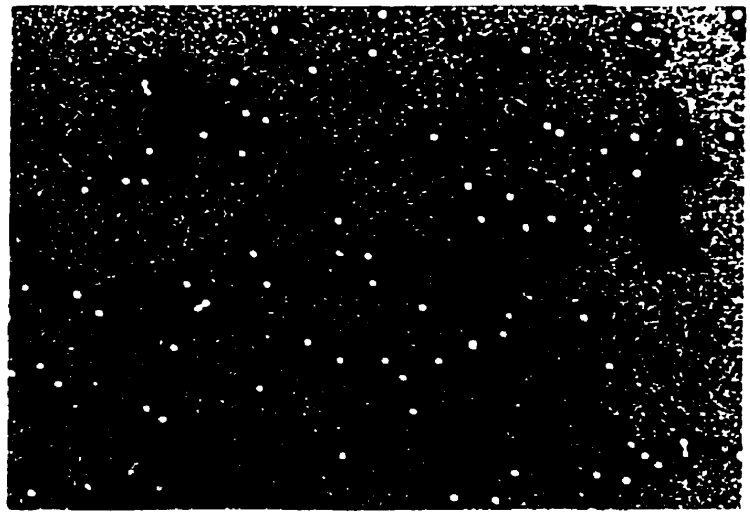

Untreated
AS 2
Migration (Fold Increase)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
20
40
ODN Concentration (μM)

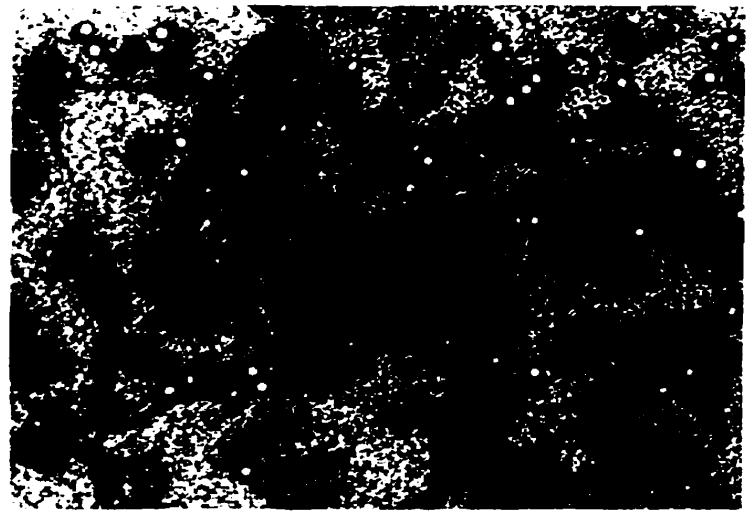
Figure 9. Light microscopy of migrating ODN-treated cells

HUVEC were treated with 5-40 μM ODN for 48 h at 37^0C. Cells were dispersed and loaded onto gelatin-coated nucleopore filters at a density of 4 x 10^4 cells/filter in Medium-199 containing 0.1% BSA. Cell migration was measured after a 24 h incubation at 37^0C. To induce cell motility, 20 ng/ml bFGF or 25 ng/ml VEGF, in 0.1% BSA were added to the lower chambers together with 10 μg/ml fibronectin. Shown are (A) untreated cells incubated in the absence of VEGF and fibronectin; (B) untreated cells in the presence of VEGF and fibronectin and (C) cells treated with 20 μM AS 2 in the presence of VEGF and fibronectin (Mag: x1000).

A



B



C

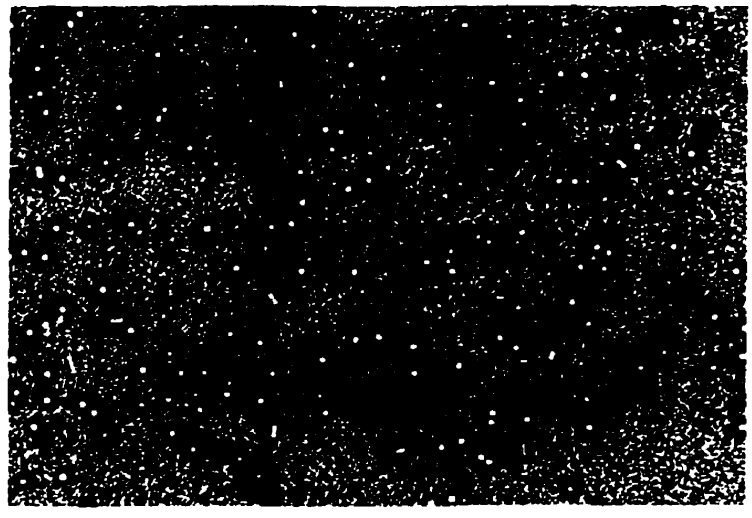


Figure 10. Inhibition of HUVEC migration by α_v antisense ODN

HUVEC were treated with ODN and seeded onto nucleopore filters as described in the legend to Fig. 7. Mouse α_v antisense ODN was used under the same conditions. Endothelial cell cultures A, B and C were isolated from three individual cords. Results are expressed as fold increase in migration relative to cells not induced by migration factors and are based on 2 filters per migration assay. Cells were used after three *in vitro* passages. The difference in the reduction caused by human and murine α_v antisense ODN was significant for endothelial cell cultures A ($p<0.0005$), B ($p<0.005$) and C ($p<0.05$).

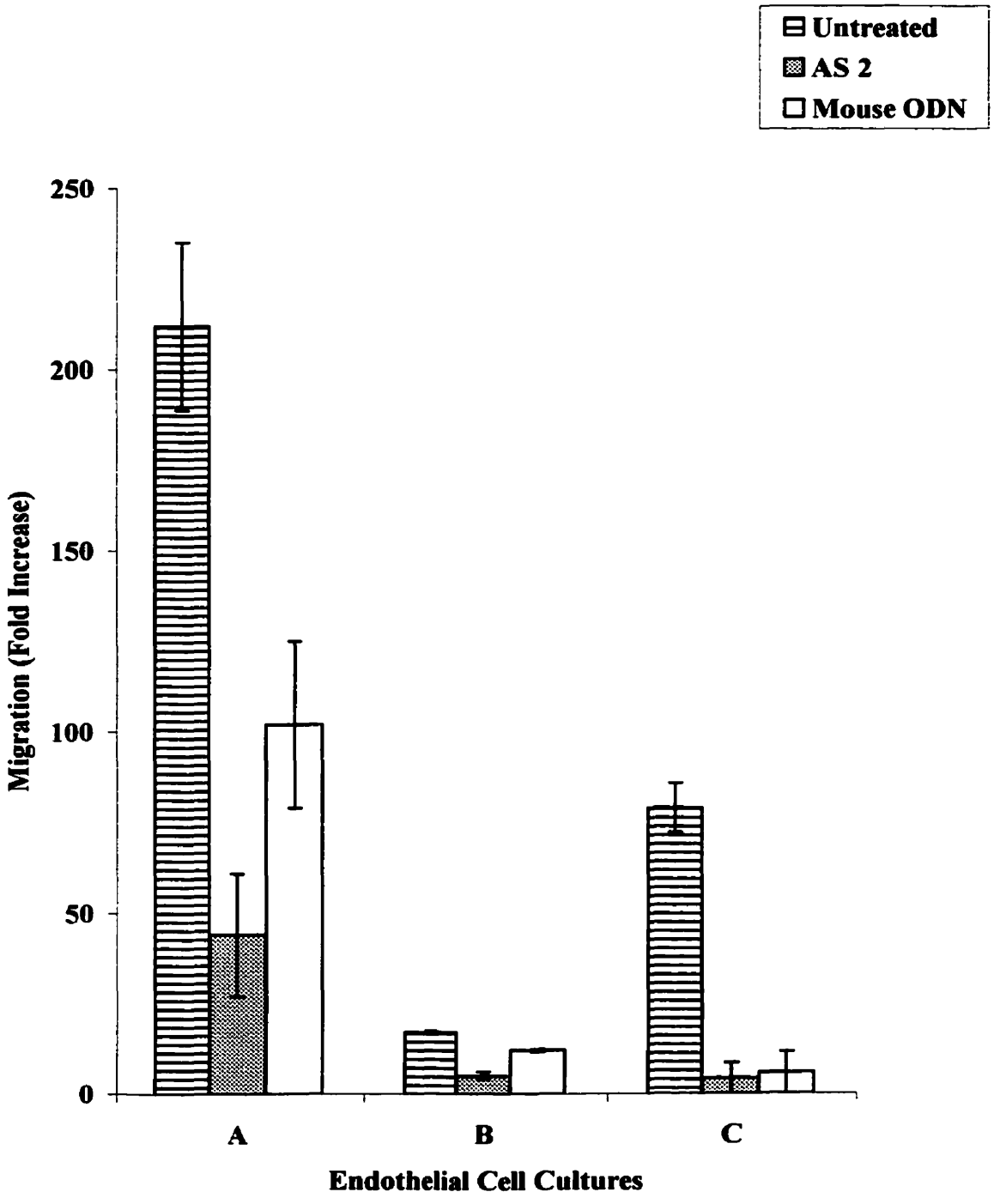
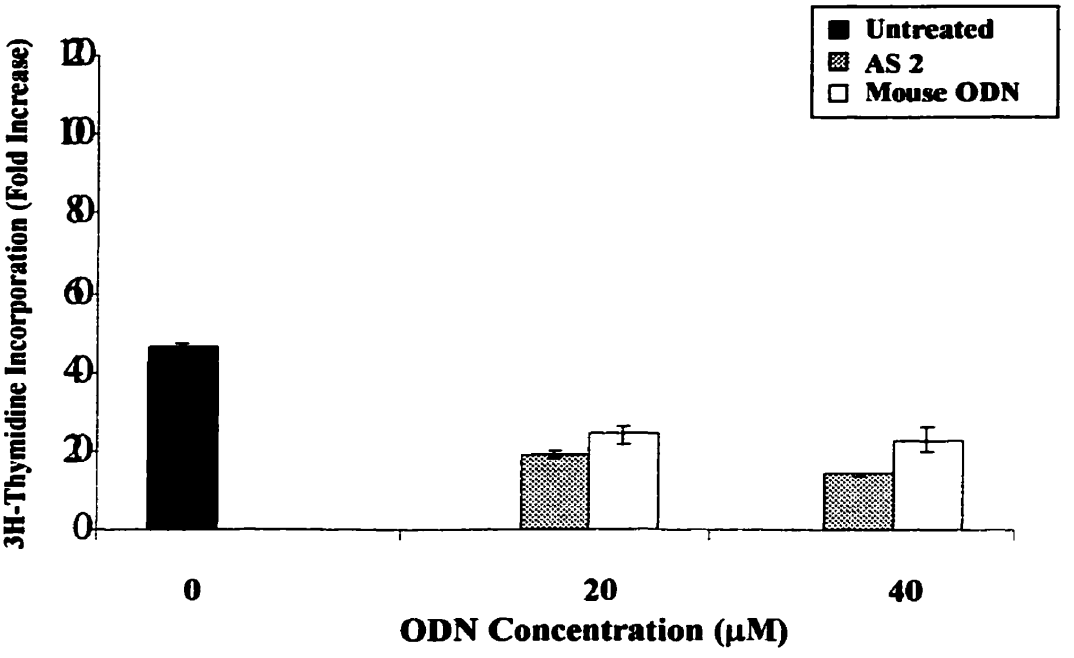

Untreated
AS 2
Mouse ODN
250
200
150
100
50
0
Migration (Fold Increase)
A
B
C
Endothelial Cell Cultures

Figure 11. Inhibition of cell proliferation by α_v antisense ODN

Assay conditions were those described in the legend to Fig. 5. ODN-treated cells were incubated for 48 (A) or 72 (B) h in the presence of medium containing 1% serum and 20 ng/ml bFGF. ^{3}H-thymidine incorporation was measured after pulsing for 18 h. Results are expressed as fold increase relative to cells cultured in the absence of bFGF and are based on experiments conducted in triplicate. The difference in inhibition caused by human and murine α_v antisense ODN at a concentration of 40 μM was significant ($p<0.05$).

A


Untreated
AS 2
Mouse ODN
3H-Thymidine Incorporation (Fold Increase)
120
100
80
60
40
20
0
0
20
40
ODN Concentration (μM)

B

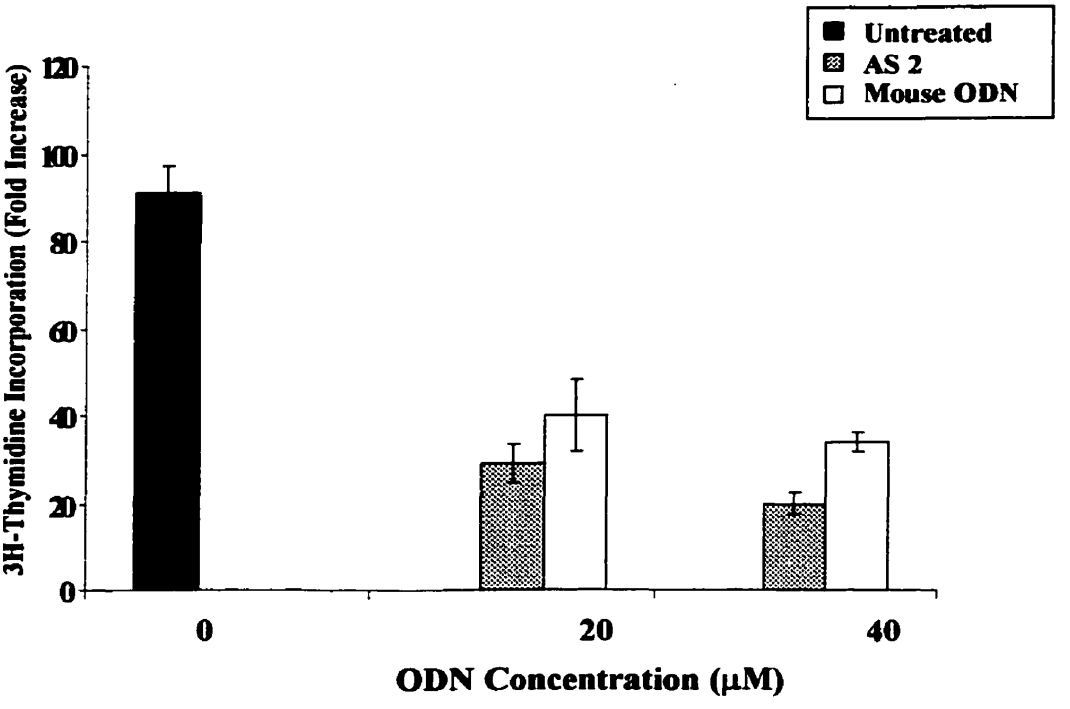
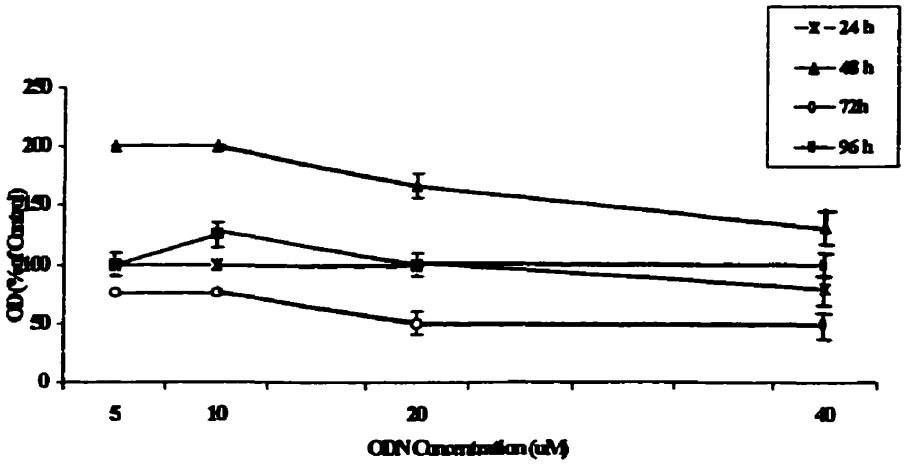

Untreated
AS 2
Mouse ODN
3H-Thymidine Incorporation (Fold Increase)
120
100
80
60
40
20
0
0
20
40
ODN Concentration (μM)

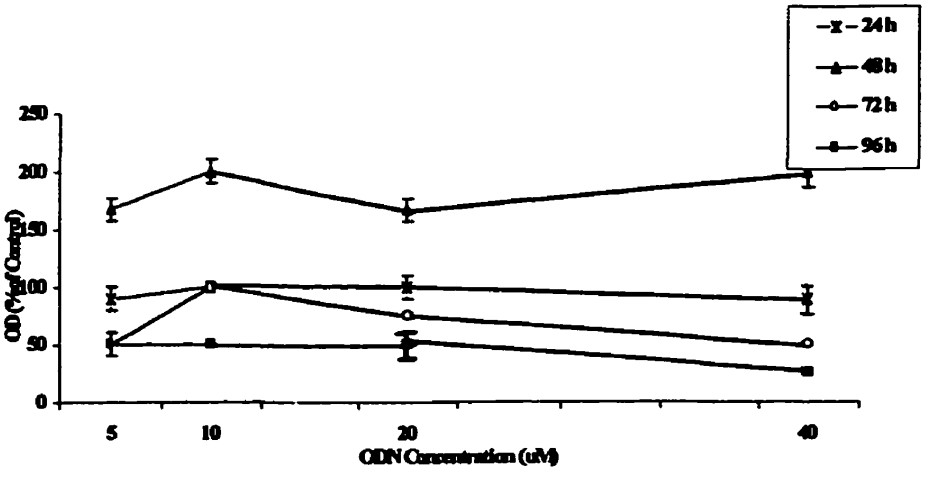
Figure 12. The inhibitory effect of α_v antisense ODN was not due to cell cytotoxicity

HUVEC were treated with 5-40 μM ODN for 48h and were seeded into gelatin-coated 96-well plates at a density of 5 X 10^3 cells/well in complete Medium-199 containing 1% FCS to mimick the conditions used for the ^{3}H-thymidine incorporation assay shown above. Cell survival was determined as described previously (see Materials and Methods). The absorbance (OD) is directly proportional to the number of viable cells. (A) Cells treated with 5-40 μM AS 2, (B) cells treated with 5-40 μM mouse α_v ODN.

A


24 h
48 h
72h
96 h
250
200
150
100
50
0
5
10
20
40

B


24 h
48 h
72 h
96 h
250
200
150
100
50
0
5
10
20
40

Chapter 4

Discussion

In the present study, primary cultures and early passages of human umbilical vein endothelial cells (HUVEC) were used to establish an *in vitro* angiogenesis assay and to assess the effect of α_v antisense oligonucleotides (ODN) on two parameters of the angiogenic process, namely cellular migration and proliferation. The present results show that HUVEC could respond to motogenic and mitogenic signals triggered by VEGF and bFGF, and that pre-treatment of the cells with α_v antisense ODN inhibited these responses.

The integrins expressed by HUVEC have been well characterized. They include $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and in particular, $\alpha_v\beta_3$ (Cheresh 1987, Luscinkas 1994). Brooks *et al.* (Brooks 1994b) identified $\alpha_v\beta_3$ as a marker of angiogenesis on vascular cells. They found that $\alpha_v\beta_3$ was abundantly expressed on blood vessels in human wound granulation tissue but not in normal skin, and that $\alpha_v\beta_3$ expression is upregulated during angiogenesis in the chick CAM model. A monoclonal antibody directed to $\alpha_v\beta_3$ (LM609) was shown by this group to block angiogenesis induced by bFGF and TNF-α. In addition, $\alpha_v\beta_3$ also potentiated blood vessel maturation in developing quail (Drake 1995). Cumulatively, these studies suggested that $\alpha_v\beta_3$ could potentially serve as an effective target for anti-angiogenic therapy, and prompted the present investigation into the potential anti-angiogenic effect of α_v antisense ODN.

Three eighteen-base antisense phosphorothioate ODN selected to have no homology with DNA sequences of other known integrin subunits, α_5 in particular, and previously shown to have α_v suppressing effects in a melanoma model, were first used. At a concentration of 40 μM, AS 2 reduced α_v expression by 78%, but AS 1 and AS 3

had no effect. This inhibitory effect was confirmed with several HUVEC cultures and was dose-dependent. A sense ODN control had no effect suggesting the reduction in α_v expresssion was due to specific effects of AS 2. The reason for the lack of effect of AS 1 and AS 3 on α_v synthesis is unknown but several mechanisms can be proposed. It is conceivable that not all sites on the target mRNA are equally accessible to ODN. This is supported by other studies. For example, Goodchild and colleagues (Goodchild 1988) using a rabbit reticulocyte cell-free translation system, tested the effect of a series of synthetic oligonucleotides complementary to the 5' non-coding and coding regions of rabbit beta-globin mRNA on endogenous protein synthesis. They found that the sites most sensitive to inhibition are at the start of the 5' noncoding region and a sequence including the initiation codon and several upstream bases. Moreover, Bacon *et al.* synthesized a series of antisense pentadecamers complementary to a variety of target sequences between the cap and AUG initiation codon regions of the c-myc mRNA. They discovered that the sensitivity of the cap-region sequences to antisense inhibition of c-myc p65 expression was 2-3 times that of the original initiation codon antisense sequence. The other target sequences downstream of the cap and up to the AUG initiation codon were comparable to the initiation codon sequence, except that the first splice junction was slightly more sensitive. At the primary initiation codon target, a dodecamer was about half as effective as the original pentadecamer, whereas an octadecamer was about twice as effective. These observations suggest that the efficacy of an antisense ODN depends greatly on the target location in a mRNA sequence. AS 2 is complementary to the sequence spanning bases 31-48 of the human α_v subunit sequence,

which encodes the signal peptide of the α_v molecule (Suzuki 1987, Fitzgerald 1987). It is possible that the corresponding α_v mRNA sequence has a higher affinity for AS 2 than those targeted by AS 1 and AS 3 although this was not the case in human melanoma cells.

Having determined the optimal conditions for HUVEC migration and proliferation, the effect of AS 2 on these two parameters was analyzed. We found that the inhibitory effect on HUVEC migration exhibited by AS 2 was dose-dependent and the reduction reached up to 95% at a concentration of 40 μM. However, we found that the ODN targeted to the mouse α_v subunit (which served as a control ODN) also decreased the cells' capacity to migrate. Although the reduction caused by the mouse control ODN was always lower than the reduction caused by AS 2, it was observed that the difference in magnitude between the reductions caused by the two ODN decreased as the concentration of the ODN increased. To confirm this observation, we repeated the experiment on three different HUVEC cultures derived from three individual cords. From this experiment, at a concentration of 40 μM, an inhibitory effect with the use of mouse control ODN was again observed but the difference in the reduction caused by AS 2 and the murine ODN was significant.

As for HUVEC proliferation, it was found that 40 μM AS 2 could reduce proliferation by up to 78%. However, an inhibition in proliferation with the use of mouse ODN was also observed but at a significantly lower magnitude of up to 62%.

We observed that individual HUVEC cultures varied significantly in respect to reductions in α_v levels resulting from α_v antisense ODN treatment. In addition,

inconsistencies in the results yielded from different experiments (i.e. α_v expression vs. migration vs. proliferation) due to the usage of different cell cultures derived from different umbilical cords were also observed. The reasons for these variable effects are presently unknown but several possibilities may be proposed. Difference in ODN uptake by different cell cultures may play some role in this variability. As ODN are negatively charged, their diffusion across the lipid bilayer of the cell membrane is highly inefficient. It has been reported that only 1-2% of the oligonucleotides are actually taken up by the cells in the absence of cationic liposomes (Bennett 1993). Cationic lipid-mediated transfection of DNA into cells is well documented and is one of the most widely used approaches for cellular delivery of oligonucleotides (Nabel 1993, Bennett 1992, Caplen 1995). This highly efficient DNA transfection technique was introduced by Felgner and colleagues (Felgner 1987). With this method, ODN complexed with lipofectin penetrate through cellular membranes, and are subsequently released in the cell cytoplasm. Because lipid-based vehicles increase efficiency of ODN delivery, they reduce both the ODN concentration and the treatment time necessary to achieve reductions in protein synthesis. We found however that the treatment of HUVEC with lipofectin in the presence of the appropriate serum-free medium had deleterious effects on the cell monolayer and caused the cells to detach from the cultured dish. The reason for this effect is not clear. Our finding that repeated addition of α_v antisense ODN in the absence of a carrier lipid resulted in reductions in α_v expression in HUVEC are consistent with results our laboratory previously obtained with human melanoma cells and also with other studies. For example, Nestle *et al.* (Nestle 1994) examined the effect of ICAM-1

antisense ODN on ICAM-1 expression in cultured keratinocytes, fibroblasts, smooth muscle cells and endothelial cells, in the absence or presence of cationic lipid (lipofectin). They found that all of these cells rapidly internalized sense and antisense compounds into the cytoplasm and that ICAM-1 expression was reduced by antisense compounds when administered with (50%) and also without (30%) lipofectin.

In addition to lipid carrier, several other approaches have been used to facilitate cellular uptake of ODN and thereby increase their efficacy. Some of the biological approaches involve viral vectors, reconstituted viruses and pseudovirions (Bunnel 1997). Other approaches include the use of polycations. The most common type of polycation is DEAE-D, which has been reported to complex with RNA molecules and enhance interferon uptake and activity (Dianzani 1971). One of the most significant and efficient physical approach used for cellular delivery of ODN is the technique of electroporation. Application of electroporation in cultured cells is well established (Grey 1992, Flanagan 1997).

Goldsmith and colleagues (Goldsmith 1984) have shown that the length of time that vascular endothelial cells are maintained in culture may profoundly affect some of their properties including prostacyclin release, angiotensin-converting enzyme activity, gene expression and cell cycle kinetics. To standardize assay conditions and increase reproducibility, HUVEC cells were used in the present study only between first and fifth *in vitro* passages, at which time they were discarded. This necessitated the use of fresh endothelial cell cultures prepared from newly obtained umbilical cords throughout this study. The inconsistency observed in the effects of α_v antisense ODN on $\alpha_v\beta_3$ expression

and function was probably due, at least in part, to variability between the cords obtained. Diverse factors such as the age of the fetus, mother's age, mother's health and clinical history, and environmental factors could have contributed to differences in $\alpha_v\beta_3$ expression, various cell properties including cell permeability, cell doubling time, expression of other relevant adhesion molecules, and growth factor and growth factor receptor levels expressed by the cells. These factors among others could have contributed to the variability both in cell surface α_v expression following antisense ODN treatment and in the functional impact of this treatment.

In vitro angiogenesis studies may be markedly facilitated by the use of established long-term human endothelial cell lines. Indeed, several laboratories recently reported on the establishment of immortalized or stable human endothelial cell lines. One such immortalized human cell line has been described by Faller *et al.* (Faller 1988). Faller and colleagues transfected human umbilical vein endothelial cells with murine sarcoma viruses and found that this resulted in a cell line which was morphologically and phenotypically unchanged and retained properties characteristic of differentiated endothelial cells (Faller 1988). In contrast to primary cultures, sarcoma virus-modified cells were able to proliferate indefinitely in culture and grew independently of supplemented endothelial cell growth factors. By microinjecting a recombinant DNA construct consisting of SV40 large T antigen and the control elements derived from the human vimentin 5' sequences (HuVim 830-T/t recombinant) into human endothelial cells, another immortalized cell line was established (Schwartz 1991). These large T antigen-expressing human endothelial cells retained several characteristics of

differentiated endothelial cells. Another stable human endothelial cell line, known as SGHEC-7, was described by Fickling *et al.* (Fickling 1992). Unlike primary endothelial cell cultures, SGHEC-7 retained a number of differentiated endothelial cell functions for up to 24 passages. Although this cell line is not "immortal", the consistency in cellular responses seen during this period makes them ideal for *in vitro* angiogenic studies.

Another problem encountered during this study was the identification of appropriate ODN controls. Due to the limited availability of mismatched and sense ODN, we have chosen to use the murine antisense ODN as the control ODN. We found that a murine α_v antisense sequence, although shared no homology with the human sequence, blocked some of the $\alpha_v\beta_3$-mediated functions and occasionally, caused a reduction in α_v levels on HUVEC. In some cases, an increase in α_v levels after treatment with murine α_v antisense ODN was seen, the reason for this observation remains unclear and needs to be addressed. The possibility that the inhibitory effect exhibited by this murine ODN was due to cell cytotoxicity was ruled out by the MTT assay. Thus, the mechanism of this inhibition is presently unclear but several possibilities should be considered. First, we found that the mouse ODN sequence has a 100% homology to a gene whose function has not yet been identified, and this particular gene is located on a human chromosome (Chromosome 22q13 BAC Clone CIT987SK-384D8). Since no further information on this chromosome or its gene product could be obtained, its role in suppressing α_v subunit cannot be explained. Secondly, it should be noted that oligonucleotides (especially chemically modified ones), like any other molecules, are capable of interacting non-specifically with other DNA sequences (Perez 1994, Burgess 1995). In some cases, these

interactions are sequence specific, while in other instances the ODN interact with molecules in a sequence-independent manner (Burgess 1995). These non-specific interactions can contribute to the overall inhibitory effect of ODN and may complicate interpretation of the data as specific and non-specific effects cannot always be distinguished. Several studies have recently demonstrated that the inhibitory activities originally assumed to be due to an antisense effect of the ODN were, in fact, attributed to non-antisense mechanisms (Stein 1996). Burgess *et al.* investigated the effect of c-myb and c-myc antisense ODN on vascular smooth muscle cell (SMC) proliferation. They found that these antisense ODN caused a significant inhibition of SMC proliferation but that this inhibition was clearly not via a hybridization-dependent antisense mechanism. Rather, the inhibition was due to the presence of four contiguous guanosine residues in the ODN sequence. This was demonstrated *in vitro* in primary cultures of SMC and in arteries *ex vivo*. They have further explored the sequence requirements of this non-antisense effect and determined that phosphorothioate oligonucleotides containing at least two sets of three or four consecutive guanosine residues inhibit SMC proliferation *in vitro* and *ex vivo*. These results suggest that the content of the ODN sequence needs to be taken into account when interpreting data derived from *in vitro* or *in vivo* use of antisense oligodeoxynucleotides. Maltese and colleagues further demonstrated that antisense specificity is determined not only by the sequence but also by its occurrence with reference to the surrounding sequences (Maltese 1995). In sum, the finding that the mouse ODN was complementary to a human chromosome whose function has not yet been identified, the content of its sequence, and its occurrence with reference to the surrounding sequences may all have contributed to the inhibitory effect demonstrated by

the mouse ODN. More studies and characterization need to be done in order to have a better understanding of the nature of this particular mouse ODN and its effects on $\alpha_v\beta_3$.

Taken together, our results strongly suggest that α_v antisense ODN could significantly reduce cell surface α_v levels on endothelial cells and inhibit cellular functions essential for angiogenesis. Several technical issues remain to be resolved including the variability caused by the use of endothelial cells isolated from different umbilical cords. This variability could probably be significantly diminished by the use of stable long-term endothelial cell lines. Other issues such as the selection of appropriate controls will need to be resolved. Lastly, studies need to be performed to confirm the effect of α_v antisense *in vivo*. Once these goals are achieved, the ODN used in this study and others to be screened, may have potential clinical applications.

The results of this study, coupled with previous reports that antagonists of $\alpha_v\beta_3$ can block angiogenesis, show that targeting of integrins may provide an effective anti-angiogenic approach. Because a large variety of adhesion molecules are expressed with great specificity on different tumor cells and at different stages of the angiogenic process (e.g. $\alpha_v\beta_3$ is only expressed upon angiogenic stimulation), it is possible to selectively interfere with these adhesive processes without blocking normal established adhesion interactions in the same tissue. Such "adhesive therapies" may represent a major new approach to the treatment of malignant tumors.

Chapter 5

Future Studies Proposed

1. Use en established stable or immortalized endothelial cell line.

2. Identify an appropriate control oligonucleotide.

3. Investigate the effect of α_v antisense oligonucleotide on angiogenesis in *in vivo* studies.

Chapter 6

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