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Phenotypic Alterations in Kaposi's Sarcoma Cells by Antisense Reduction of Perlecan

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Metastasis is a sequence of events including proliferation, migration, adhesion, invasion and subsequent metastatic growth of tumour cells in distant organs. We previously showed that highly metastatic variants of murine melanoma cells express higher levels of the basement membrane proteoglycan perlecan than low or non metastatic variants and expression of an antisense perlecan can reduce metastatic potential. In contrast, antisense expression of perlecan in fibrosarcoma cells was reported to enhance tumorigenesis. To better understand the role of perlecan in angiogenesis we have transfected KS-IMM, an immortalized cell line derived from a human Kaposi's sarcoma, with an antisense perlecan construct and investigated the positive/negative role of perlecan in KS. KS-IMM cells were transfected with either empty vector (neo) or the antisense perlecan construct and clones were isolated. Immunoblot analysis showed a reduction of perlecan levels in two (AP3 and AP4) isolated clones, in Northern blot analysis endogenous perlecan was undetectable in the AP3 and AP4 clones, while it was present in the neo control clones. AP clones had a reduced migration to HGF in Boyden chambers as compared to neo clones. Proliferation in low serum or serum-free conditions was strongly reduced in the AP clones as compared to the neo control cells. The neotransfected cells showed rapid proliferation in low serum supplemented with HGF and VEGF, while antisense transfected clones showed little response. Finally, AP-trasfected KS-IMM cells had significantly reduced migration to VEGF and HGF with respect to controls. In contrast, when the AP transfected cells were injected in nude mice they paradoxically showed enhanced tumor growth as compared to controls. Our preliminary data indicate that perlecan reduction plays a crucial role on Kaposi's sarcoma cell migration and proliferation in vitro. However, in vivo KS-IMM depleted of perlecan had a growth advantage. A possible hypothesis is that perlecan is necessary for growth of KS-IMM cells in vitro, however its down-regulation might promote angiogenesis through increased angiogenic growth factor diffusion, resulting in enhanced tumor growth in vivo. (Pathology Oncology Research Vol 6, No 1, 10–17, 2000)

Keywords: Kaposi-sarcoma, perlecan, antisense

Introduction

Kaposi's sarcoma (KS) is a highly angiogenic lesion found with increased frequency in AIDS patients.[5] KS is associated with immune suppression and infection with herpes virus HHV-8,[30] however the link between these components and KS is still unclear.[15] KS lesions principally consist of newly formed, abnormal vessels, an inflammatory infiltrate and proliferating spindle-shaped cells that are generally considered the malignant component of KS. The origin of KS spindle cells is still debated; these cells simultaneously express markers for endothelial cells, macrophages and mesenchymal cells, leading to suggestions that they are derived from a primitive vascular precursor or "dedifferentiated" endothelial cells.

The HIV Tat protein along with bFGF are thought to cooperate as exogenous factors stimulating KS.[12] KS cells them-

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selves produce several angiogenic factors, prominent among these are VEGF[10,22] and HGF.[20,24] Like Tat with bFGF, HGF has been reported to stimulate cultured endothelial cells to assume a spindle-like morphology in culture reminiscent of KS cells.[24] Production of VEGF by KS cells, which are positive for the VEGF receptor KDR, appears to contribute to an important autocrine loop for KS.[22] Infection by HHV-8 appears to contribute initialization of this autocrine loop, as HHV-8 infection of endothelial cells in vitro stimulates VEGF production and up-regulates KDR expression.[14]

The heparin binding growth factors VEGF, HGF and bFGF require heparin or heparan sulfate as a cofactor for presentation to their respective receptors and induction of signalling events.[28,29,33,35] In vivo it is the heparan sulfate proteoglycans (HSPGs) on the cell surface and in the extracellular matrix which act as the cofactor in HBGF-receptor interactions. HSPGs are ubiquitous macromolecules associated with the cell surface and the extracellular matrix of most all cells and play central role in embryonic morphogenesis, angiogenesis, neurite outgrowth and tissue repair.[19,34] These proteoglycans can have several different core proteins which determine many characteristics of the molecule, including expression, localization, interactions with other proteins and possibly even a different degree of heparan sulfate side chain modification.

Recently, substantial evidence indicates that some heparin-binding growth factors may require a specific proteoglycan for presentation to their receptors.[6,8] Specific down regulation of the proteoglycan involved blocks the cellular response to the growth factor,[1,7] while over expression of other proteoglycans could also inhibit HBGF-receptor interactions.[21,36] In fibroblasts it has been shown that perlecan, an extracellular matrix HSPG, is very efficient in presentation of bFGF to its receptor, while in contrast, over-expression of the cell surface HSPG syndecan-1 resulted in inhibition of bFGF responses. However, there is also evidence suggesting that the proteoglycan specificity may change from cell type to cell type; a reduction of perlecan in HT1080 fibrosarcoma cells favoured tumor growth,[23] while the cell surface HSPG syndecan-1, -2, -4 and glipican-1 were effective in promoting bFGF-receptor interactions in K562 cells.[32] Different growth factors may also vary in dependence on heparan sulfate, for example FGF-7 binds the perlecan core protein independently from its heparan sulfate chains, suggesting a key role for the core protein in FGF-7/receptor interactions.[31] These data indicate that HSPGs might have different regulatory potential on tumor and host cells of diverse origins.

Recent findings indicate that Kaposi's sarcoma cells possess the KDR, Flt-1 and Flt-4 VEGF receptors.[22] In addition, KS cells express the c-*met* receptor for HGF.[20] Both VEGF and HGF are thought to play key roles in Kaposi's sarcoma development and maintenance.[10,20,22,24] Here we show that anti-sense reduction of perlecan synthesis by KS cells results in a reduced growth in low serum. Reduction of perlecan was also associated with a reduction in the growth and migratory response to VEGF and HGF by these cells. In contrast, however, the antisense perlecan cells formed larger and more rapidly growing tumors than controls, apparently due to increased diffusion of the angiogenic factor produced by these cells.

Materials and Methods

Antisense Construct

A segment of the human perlecan cDNA corresponding to 1.1 kb of the 5' end comprising domain I was amplified with the primers5'-CATAggATCCATggg CTgAgggCATACgA-3' and 5'-TTATAAgCTTCgCTCTCCTCgTCACAgTg-

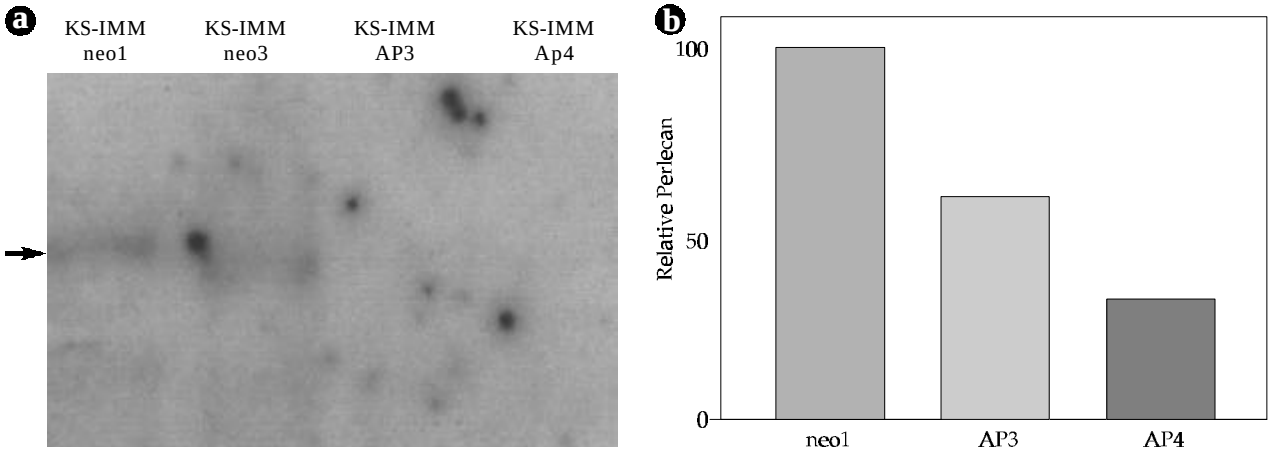

Figure 1. (a) Northern blot analysis of total RNA isolated from AP3, AP4, neo1 and neo3 transfected cells. Endogenous perlecan mRNA is present in the neo controls while the perlecan mRNA was not detected in the AP transfected cells. (b) Release of perlecan into the conditioned medium of neo and transfected AP cells as assesed by an immunoblot assay followed by desitometry of the intensity of staining. Antibody reactivity with serial dilutions of secreted proteins demonstrated a reduced production of perlecan of approximately 50% in AP3 and approximately 70% in AP4 cells with respect to the neo1 clone.

gAA-3' using RT-PCR. The amplified cDNA was cloned into the pHβAP-neo1 vector.[16] in the antisense orientation by digestion of the vector and the amplified cDNA with HindIII and BamHI. Plasmids were isolated and the orientation confirmed by restriction mapping. This antisense perlecan plasmid vector was termed pAP.

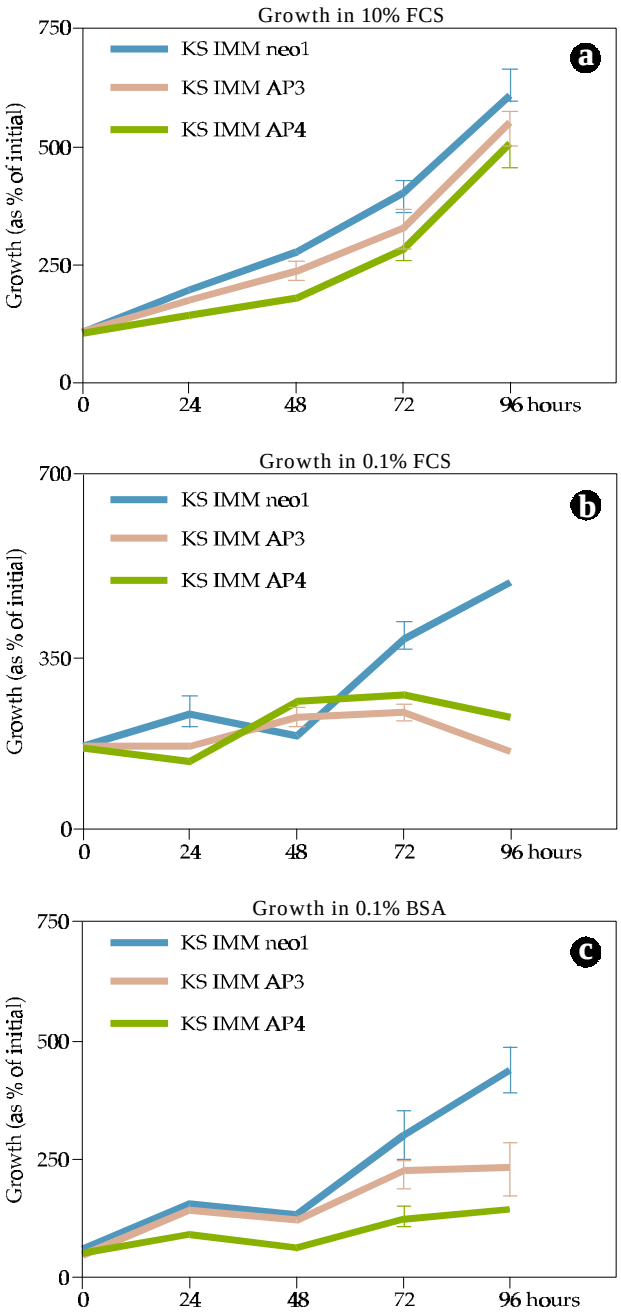

Figure 2. Growth of AP3, AP4 and neo1 KS IMM transfected cells under limiting conditions. No significant differences were noted between the neo1 control cells and the AP clones in normal growth conditions with 10% FCS (a). However in 0.1% FCS (b) and serum-free media with 0.1% BSA (c) the AP cells showed strongly reduced growth.

Cell culture and transfection

The human Kaposi's sarcoma immortalized cell line KS-IMM[3] was maintained in DMEM with 10% FCS supplemented with Glutamine (300 µg/ml). KS-IMM cells were transfected by the calcium phosphate method with either the antisense perlecan construct pAP or the pHβAP-neo1 vector alone as a control using 20 µg of phenol-chloroform purified plasmid DNA. The transfected cells were selected for G418 resistance by culture in media supplemented with 0.4 mg/ml G-418, resistant colonies was isolated and 6 clonal lines obtained for both anti-sense perlecan and vector alone transfectants. These lines were maintained under G418 selection and termed AP 1 through 6 and neo 1 through 6, respectively.

Northern blot analysis

RNA was extracted from confluent flasks of AP or neo transfected cells with the method of Sacchi and Chomczynski.[9] For Northern hybridization, 15 µg of RNA denatured and mixed with 1mg/ml Ethidium bromide was eletrophoresed on 0,7% agarose gel containing formamide. The RNA was transferred to a nylon membrane (Hybond-N Amersham) and hybridized using a 1.1 kb human perlecan cDNA ^{32}P-labelled probe.

Immunoblot assay

The antisense human perlecan and vector alone transfected cells at confluence were incubated 24 hours with DMEM without FCS. The conditioned medium was collected, pretreated with protease inhibitors (Leupeptin, A-protinin, Pepstatin and PMSF), and assayed for total protein content using the Bradford Dye Reagent method (Biorad). The samples were equalized for the protein content and serially diluted (1:2), spotted and absorbed to a Nitrocellulose membrane. Nonspecific binding sites were blocked for 1 hour at 20C in PBS with 2% BSA. The blot was then incubated with an antiperlecan antibody (1:1000 dilution in PBS-2%BSA). Subsequently the blot was washed with PBS 0.05% Tween 20. The positive signal was detected by ECL (Amersham) and visualized by fluorography. Quantitative protein analysis was performed by densitometric scanning and the NIH Image program.

Chemotaxis assay

The chemotaxis assay was performed as previously described.[2] Briefly, AP and control neo transfected cells ($1{,}3x10^5$ cells) were seeded in serum free RPMI supplemented with 0.1% BSA into the upper chamber of a modified Boyden chamber separated by a gelatin coated PVP

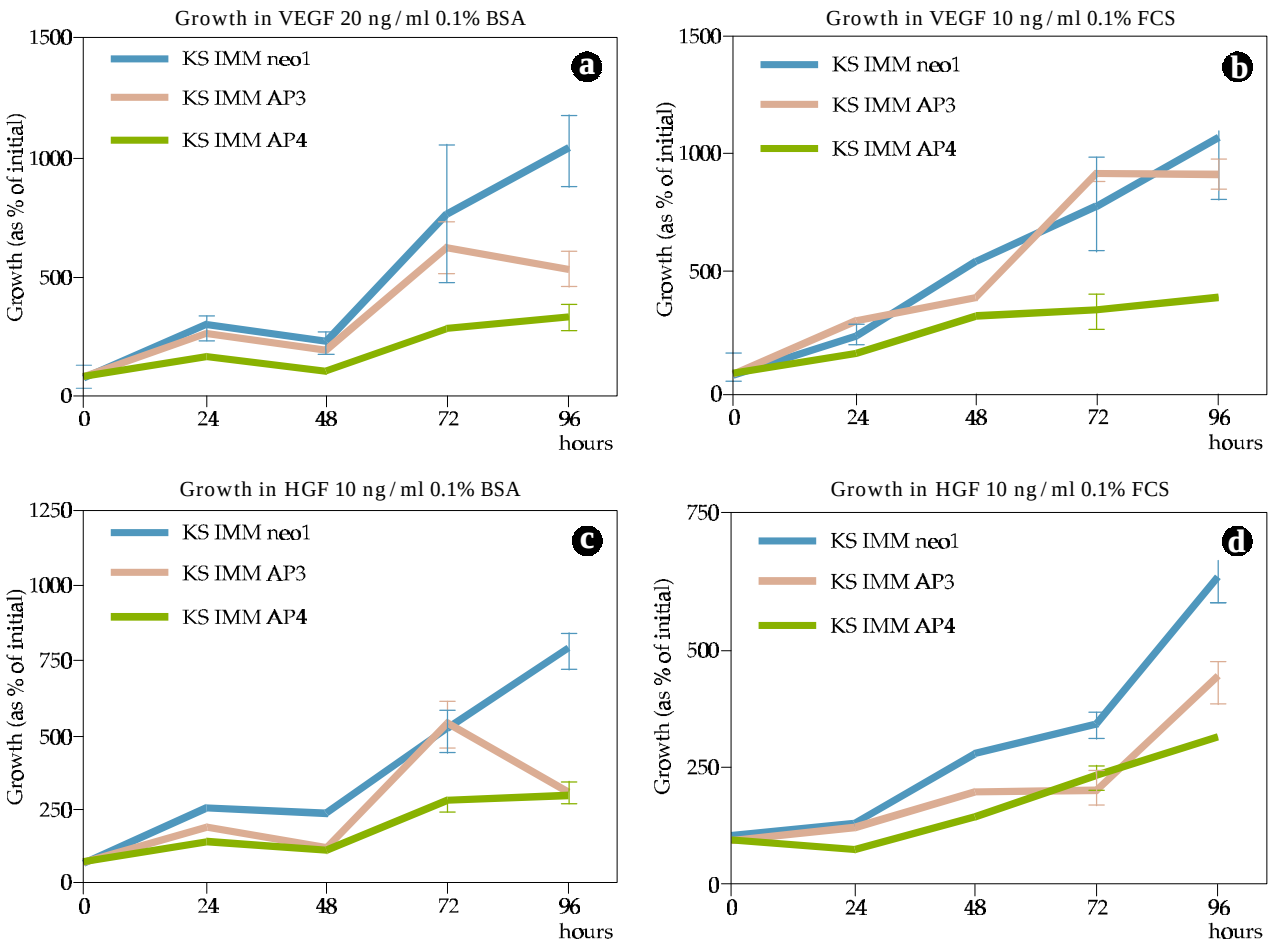

Figure 3. Growth of AP3 and AP4 cells in response to VEGF or HGF as compared to neo control clones. In 0.1%BSA both the AP3 and AP4 cells demonstrated reduced growth in presence of VEGF (a) or HGF(c). In presence of 0.1%FCS and 10ng/ml of VEGF the AP4 transfectant continued to show growth inhibition while no difference was observed between AP3 and neo1 clones (b).

free polycarbonate filter (Nucleopore). As a chemoattractant in the lower compartment of the Boyden chamber either serum free RPMI supplemented with 0,1% BSA (SFM, a control for background migration) or SFM supplemented with VEGF (10 ng/ml or 20 ng/ml) or HGF (5 ng/ml or 10 ng/ml) were used.

After six hours incubation at 37°C in a humidified 5% CO2 atmosphere, the cells remaining on the upper surface of the filter were removed mechanically and the migrated cells on the lower surface stained with Toluidine Blue and counted with a microscope.

Cell proliferation assay

AP and neo transfected cells were plated in a 96 well microtiter plate (800 cells/well) in complete DMEM medium for 24 hours. After 24 hours, media were removed and into different wells D-MEM supplemented with either 10% FCS, 0.1%FCS or 0.1% BSA were added. In some wells the low serum or serum-free media were also supplemented with either VEGF or HGF in order to assay if perlecan affected proliferation in response to these growth factors. Cell growth was determined using the metabolic MTT assay at 24 hour periods over 4 days.

In vivo tumor growth

To investigate the effects of perlecan reduction on tumor growth in vivo, $5x10^6$ AP3, AP4, neo3 or neo1 cells were inoculated in nude mice in vivo, either suspended in matrigel or in media alone. Six animals were used for each group. Tumor size was measured regularly, and the animals were sacrificed 20 days after inoculation, the tumors weighed and collected.

Each sample was fixed in formalin, embedded in paraffin and histologically examined. From each sample DNA was also extracted and the presence of the antisense construct demonstrated by PCR.

RESULTS

Antisense perlecan transfection

KS-IMM cells were transfected with the pHβAP expression vector containing a 1.1 kb perlecan cDNA in the antisense orientation (pAP) or with the vector alone. Northern blot analysis of total RNA isolated from the transfected KS-IMM cells showed expression of the endogenous perlecan mRNA in the neo controls, while the perlecan mRNA was undetectable in the AP3 and AP4 transfected cells (*Figure 1a*). The reduction of perlecan expression was confirmed by an immunoblot assay with the conditioned media from the neo and AP transfected cells using a polyclonal anti murine perlecan antibody. Antibody reactivity with serial dilutions of secreted proteins demonstrated reduced perlecan in the two anti-sense transfected clones, AP3 and AP4, as compared to the neo KS-IMM cells (*Figure 1b*).

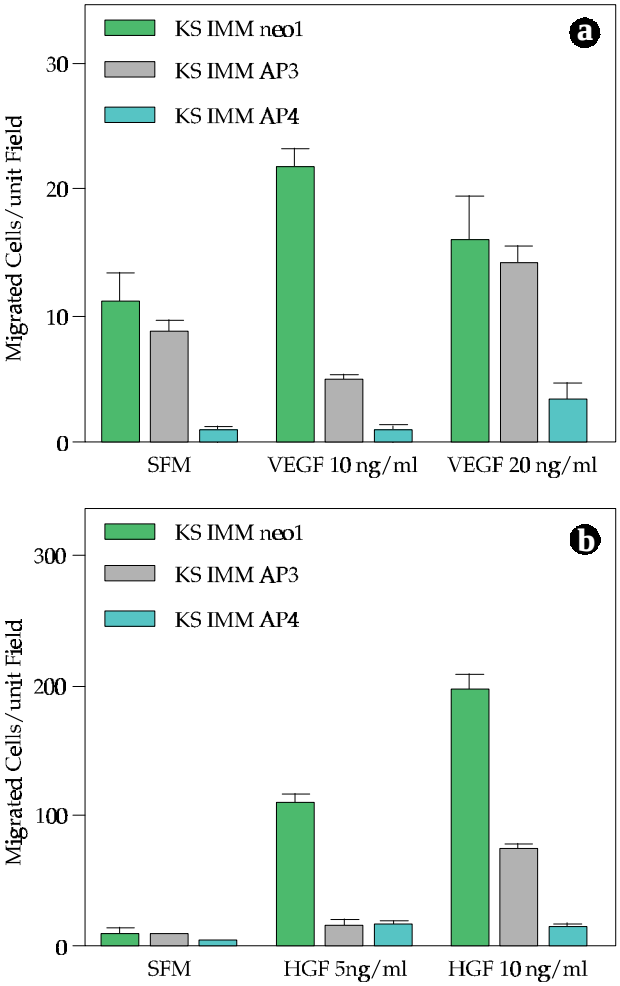

Figure 4. Chemotaxis of the AP3, AP4 and neo1 clones towards VEGF or HGF. Serum free media was used alone as positive control or supplemented with growth factor as chemoattractant. Control neo transfected cells showed a migratory response towards both 10 and 20 ng/ml VEGF(a). In contrast, the AP4 cells were unable to respond to VEGF, while the AP3 cells responded to the higher concentration of VEGF (a). AP4 cells were also unable to respond to HGF (b) while the AP3 cells showed a partial response at the higher concentration of HGF (b)

Growth in vitro

KS cells express receptors for VEGF, KDR/flt-1[10,22] and HGF, c-met,[20,24] and both autocrine and paracrine loops may be involved in the growth and invasion of KS-IMM cells. We therefore tested the growth response of these cells under growth factor limiting conditions and in response to specific growth factors. No significant differences were noted between the control neo and the perlecan antisense transfected cells AP3 and AP4 in normal growth conditions with 10% FCS in the media. However, in low serum (0.1% FCS) both the perlecan deleted cells showed strongly reduced growth, while the control transfectants replicated well beyond 48 hours in culture (*Figure 2*). Similar growth characteristics were observed for cells grown in serum-free conditions (0.1% BSA), where again the controls replicated well after 48 hours, in contrast to the perlecan deleted cells. These data suggested that the reduction in perlecan expressed by these cells could have interrupted an autocrine loop present in the parental cells.

Cell growth in the presence of individual growth factors was then assessed in either low (0.1% FCS) serum or in serum-free conditions (0.1% BSA). In serum-free conditions both the AP3 and AP4 transfectants demonstrated reduced growth in the presence of VEGF or HGF, with AP4, the transfectant expressing the least perlecan, having the greatest inhibition *(Figure 3).* In the presence of 0.1% FCS, the AP4 transfectant continued to show growth inhibition, while no difference was observed between AP3 and the neo control. Both antisense transfected lines showed limited growth in HGF with 0.1% FCS.

Chemotaxis in vitro

The ability of the transfected cells to respond to the growth factors VEGF and HGF was tested using the chemotaxis assay. Serum-free media (with 0.1% BSA) was used alone (as the negative control) or supplemented with specific growth factors as chemoattractants. Control neo transfected cells responded well to both 10 and 20 ng/ml of VEGF. In contrast, the AP4 cells were unable to respond to VEGF, while AP3 cells were able to respond to higher concentrations of VEGF *(Figure 4).* Control neo transfected cells responded dose-dependently well to HGF. Again, the AP4 cells were unable to respond, while AP3 cells showed a partial response at higher concentrations. These data suggest that loss of perlecan resulted in a block of the ability to respond to either VEGF of HGF.

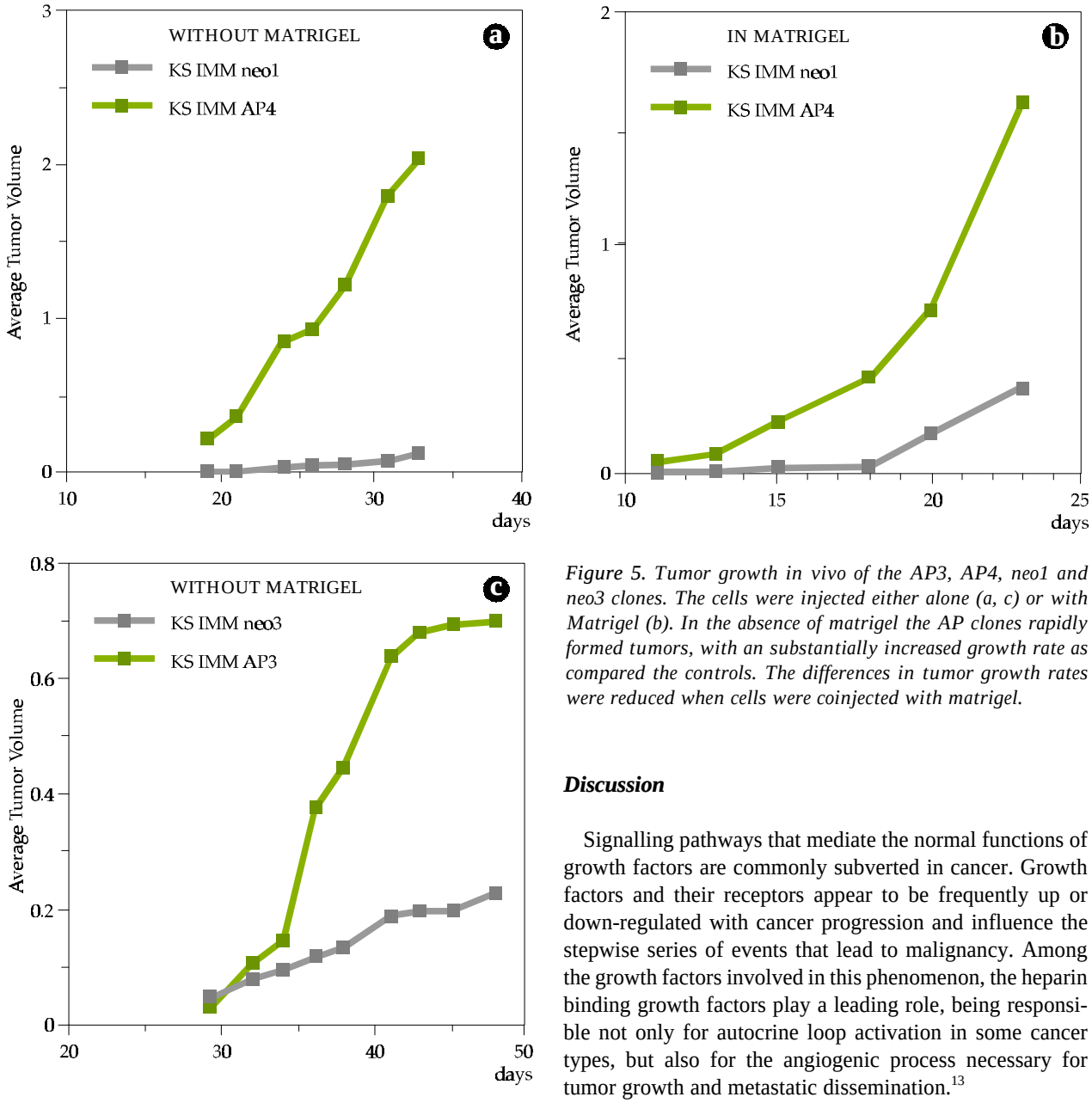

Figure 5. Tumor growth in vivo of the AP3, AP4, neo1 and neo3 clones. The cells were injected either alone (a, c) or with Matrigel (b). In the absence of matrigel the AP clones rapidly formed tumors, with an substantially increased growth rate as compared the controls. The differences in tumor growth rates were reduced when cells were coinjected with matrigel.

Tumor growth in vivo

The effects of reduction of perlecan synthesis were tested by assessing tumor growth *in vivo*. The AP3 or AP4 clones were injected subcutaneously in nude mice, and the growth or tumors was compared to growth either neo1 or neo3 control clones injected at the same time. Tumor growth was tested with cells injected either alone or in the presence of the basement membrane extract Matrigel. In the absence of matrigel, the AP clones rapidly formed tumors with an increased growth rate as compared to the control neo transfected cells (*Figure 5*). The differences in tumor growth rates were substantially less when matrigel was used as a vehicle for injection.

Discussion

Signalling pathways that mediate the normal functions of growth factors are commonly subverted in cancer. Growth factors and their receptors appear to be frequently up or down-regulated with cancer progression and influence the stepwise series of events that lead to malignancy. Among the growth factors involved in this phenomenon, the heparin binding growth factors play a leading role, being responsible not only for autocrine loop activation in some cancer types, but also for the angiogenic process necessary for tumor growth and metastatic dissemination.[13]

Although several studies have documented the interactions of heparin binding growth factors with heparin, in some cases defining the actual sequence within the variable heparin polymer which the factor binds to, relatively little study has been dedicated to defining the heparan sulfate proteoglycans with which these growth factors interact in vivo. Significant data exists to suggest that there is usage of specific heparan sulfate proteoglycans in cell-growth factor interactions.[6-8] This proteoglycan usage specificity appears to give an entire new level of regulation of growth factor activity.

Studies in fibroblasts indicate that the HS proteoglycan perlecan is necessary for bFGF-receptor interaction. A scan of a series of purified HS proteoglycans from human

lung fibroblasts indicated several proteoglycans showed little activity, including Syndecans 1 and 2, and glipican 1, while perlecan showed a potent ability to to mediate bFGF-receptor interactions.[6] The key role for perlecan in these interactions is also suggested by studies in which syndecan 1 was over-expressed in fibroblasts, resulting in a blockage of the ability of these cells to respond to bFGF, apparently by binding bFGF but not presenting it to its receptor.[21,36] The overexpression of an antisense perlecan cDNA in fibroblasts significantly reduced perlecan expression.[7] These cells were also unable to respond to bFGF, even though there were no changes in bFGF receptor number, overall heparan sulfate proteoglycan synthesis or sulfation of the heparan sulfate. Addition of exogenous heparin or perlecan restored the response. These data suggested that there is a strict dependence on perlecan for fibroblast-bFGF interactions.

A similar effect was noted in melanomas.[7] We have recently demonstrated that a reduction of perlecan expression using our antisense strategy in murine melanoma reduced the invasive and metastatic potential of these cells.[1] There are indications that the role for perlecan could be related to cell type, however. Syndecans and glipican were found to be effective in bFGF-receptor presentation in K563 cells,[32] and perlecan reduction in HT1080 fibrosarcoma cells led to increased invasion.[23] The significance of the role for proteoglycans in determining cellular responses to growth factors has been shown in developing systems. The aFGF to bFGF switch seen in neurons is controlled by changes in the presenting proteoglycan, an alternate splice variant of perlecan, and not by either growth factor or receptor availability.[18,27] Elimination of perlecan by gene targeting resulted in an embryonic-lethal phenotype with failure of neural tube closure, exencephaly and severe skeletal malformations.[4,11] The skeletal defects were similar to those in which FGF3-receptor interactions are perturbed,[4] suggesting that perlecan may be critical for FGF3-receptor interactions in developing cartilage.

The expression of both ligand, receptor and high levels of the presenting proteoglycan would lead to constitutive activation of a proliferative signal in tumor cells. These loops could confer acquisition of the invasive and angiogenic phenotype. Some tumor types often show an increase in the synthesis of specific proteoglycans. We have observed that clonal cell lines from a murine melanoma with an invasive, metastatic phenotype showed significantly higher perlecan expression than that of non-invasive, low metastatic clones of the same parental line.[25,26] In contrast, syndecan 1 expression is often decreased in malignancies.[17] These data suggest that perlecan may be an effective mediator of growth factor – receptor interactions in some tumor cell types, whereas syndecan 1 could be a suppressor of these interactions. Here we have shown that reduction of perlecan expression in Kaposi's sarcoma cells results in an inhibition of the responses by these cells to VEGF and HGF. In contrast, tumor growth was accelerated in the same perlecan-reduced cells. One possibility is that reduction of perlecan interferes with autocrine loops in vitro, but results in greater diffusion of these angiogenic growth factors in vivo, resulting in increased angiogenesis and tumor expansion. For most tumor types the use of the basement membrane extract Matrigel, which contains perlecan, results in improved tumor take and growth. In contrast, the growth of the AP KS-IMM cells was more similar to that of the neo transfected control cells when injected with matrigel. These data suggest that exogenous matrigel partially compensated for the AP phenotype.

The role of angiogenesis in the increase in tumor growth of AP transfected cells is not yet clear. No alterations of early vasculogenesis in embryonic development as determined by PECAM staining were noted in the perlecan deleted mice.[11] However, perlecan may play a key role in vessel formation at later stages, as microaneurysms were reported in perlecan negative embryos at later developmental stages.[11] Perlecan may also play a role in tumor angiogenesis in the adult host, our data suggest that perlecan affects the autocrine and paracrine growth effects of VEGF and HGF in KS cells in vitro, but that KS cell growth in vivo is favored by lowered perlecan. This latter effect may be due to increased diffusion of angiogeneic factors released by the KS cells into the host tissues.

Acknowledgement

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