An Angiogenic Role for the Neurokines Midkine and Pleiotrophin in Tumorigenesis

Rangana Choudhuri, Hua-Tang Zhang, Sandra Donnini, Marina Ziche, and Roy Bicknell

Molecular Angiogenesis Group, Imperial Cancer Research Fund, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom [R. C., H.-T. Z., R. B.]; and Department of Pharmacology, University of Florence, Viale G. B. Morgagni 65, 50134 Florence, Italy [S. D., M. Z.]

ABSTRACT

Recent analysis of bladder tumors has correlated expression of the neurokine midkine (MK) with poor patient survival. To examine a role for MK and the related pleiotrophin (PTN) in tumorigenesis, they were overexpressed in MCF-7 breast carcinoma cells. Expression had no effect on in vitro growth but conferred a growth advantage in vivo. Enhanced proliferation, implicating an angiogenic role for MK and PTN. Angiogenic activity of MK and PTN was confirmed in the rabbit corneal assay. Our data therefore identify two novel targets for antiangiogenic drug development.

INTRODUCTION

MK (a 13-kDa polypeptide) and PTN (a 15-kDa polypeptide) are secreted heparin-binding neurokines that share 50% sequence homology (1). The expression of MK and PTN had initially been considered to be restricted to embryonic development and within the adult brain (2-5). Recently, however, MK and PTN have also been shown to be expressed in a range of primary human tumors (6-9). For example, we have demonstrated that expression of MK in invasive bladder carcinomas correlates with poor patient survival (10). Furthermore, inhibition of PTN expression in experimental melanomas using hamster ribosomes reduced tumor formation in nude mice, indicating a direct role for PTN in tumorigenesis (11). Nevertheless, the mechanisms by which PTN stimulated melanoma growth and MK adversely affected patient survival have not been addressed.

The purpose of the studies described here was to attempt to elucidate the role (if any) of expression of MK and PTN in tumorigenesis. We have shown by transfection and xenograft experiments that expression of either MK or PTN enhances tumor growth, endothelial proliferation, and vascular density. In the light of this, we postulate that these factors mediate angiogenesis in the tumor and have confirmed this by showing that expression of MK and PTN in MCF-7 cells induces a strong angiogenic response in the rabbit corneal assay.

MATERIALS AND METHODS

Materials. MDA-435S (ATCC HTB-129) cells were obtained from the ATCC (Bethesda, MD). MCF-7 cells (passage 53) were a gift from Dr. Marc Lippman (Georgetown University, Washington, DC). BALB/c 3T3 fibroblasts (ATCC CCL 163) were supplied by the ICRF (Cell Production Service, London, United Kingdom). Female BALB/c-nu/nu mice were bred at the ICRF, Clare Hall Laboratories and used when 6-8 weeks old. Mice were maintained and manipulated in accordance with the British Home Office Animals Act, 1986. HUVECs were isolated as described and used in experiments up to passage 4 (12). A full-length mouse MK cDNA was from Professor J. Heath (University of Birmingham, United Kingdom). Full-length human PTN cDNA was from Dr. P. Milner (Washington University Medical Center, St. Louis, MO). Polyclonal goat anti-PTN antibody was obtained from R & D Systems (Abingdon, United Kingdom). Rabbit anti-MK serum was a gift from Professor T. Muramatsu (Nagoya University School of Medicine, Japan). Monoclonal rat anti-mouse CD31 antibody, MEC 13.3 was a gift from Professor A. Mantovani (Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy). Sixty-day slow release pellets containing 1.5 mg of 17-estradiol were obtained from Innovative Research of America (Toledo, OH).

Cell Culture. Cell lines were routinely cultured in HEPES-buffered DMEM supplemented with 10% FCS, penicillin (100 μg/ml), streptomycin (100 units/ml), and glutamine (2 mM). HUVECs were cultured in the same media supplemented with basic fibroblast growth factor (10 ng/ml). All cultures were Mycoplasma free and maintained in a humidified atmosphere of 5% CO2/95% air at 37°C.

Transfection of MCF-7 Cells. A single-cell MCF-7 suspension (1 x 107 cells) in early log growth phase was incubated on ice for 30 min with 40 μg of supercoiled plasmid DNA in 272 mM sucrose, 7 mM sodium phosphate (pH 7.4), and 1 mM MgCl2. Cells received a single pulse of 400 V/25 μF, were allowed to sit at room temperature for 10 min, and then on ice for another 10 min. Cells were seeded and selected 2 days later with 500 μg/ml G418.

Immunoblotting. Media (20 ml) from 107 confluent MCF-7-transfected cells was collected 2 days after feeding. Heparin-agarose (1 ml; Bio-Rad) was added to the conditioned media and shaken overnight at 4°C. The agarose was collected by centrifugation and washed with PBSA (X3). The agarose was then suspended in 2 m NaCl (1 ml) and shaken for 4 h at 4°C. The agarose was removed by filtration with a 0.25-μm filter and the flow-through was desalted using an Amicon-10 concentrator. Following SDS-PAGE (15% acrylamide gel), immunoblotting was performed as detailed (13) with minor modifications. Briefly, proteins were transferred overnight to Immobilon-P membrane (Millipore, Bedford, MA) in 25 mM Tris base, 150 mM glycine, and 15% methanol (pH 8.3) at 25 V in a Bio-Rad Mini-Transblot apparatus according to the manufacturer’s instructions. Membranes were blocked for 2 h in PBSA, 0.1% Tween 20, and 5% Marvel fat-free milk. To detect MK, rabbit anti-MK polyclonal serum was diluted 1:1000 in blocking buffer and applied to the membrane at 4°C overnight. The membrane was then washed in blocking buffer and incubated with biotin-conjugated mouse anti-rabbit (DAKO Ltd., High Wycombe, United Kingdom) IgG diluted 1:1000 for 1 h at 4°C. The membrane was treated with streptavidin horseradish peroxidase (Amersham International, Amersham, United Kingdom) diluted 1:1000 for 1 h at 4°C. Proteins were visualized by enhanced chemiluminescence (Amersham). PTN was detected in the same way using goat anti-PTN polyclonal at a concentration of 2 ng/ml followed by biotin-conjugated mouse anti-goat IgG.

Endothelial Cell Proliferation Assay. HUVECs were seeded at 103 cells per well in HEPES-buffered DMEM containing 10% FCS and 10 ng/ml basic fibroblast growth factor. The next day cells were refed with media lacking basic fibroblast growth factor but containing 60 μl of 1 ml heparin-agarose eluate containing between 10 and 50 ng of MK or PTN. Three days later, the cells were released by treatment with trypsin/EDTA and counted in a Coulter counter.
Angiogenesis Assay and Xenograft Experiments. Corneal angiogenesis assays were performed in female New Zealand White rabbits using a standard protocol (14) in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC law no. 86/609). Albino rabbits received in the left cornea $2.5 \times 10^5$ or $3.6 \times 10^5$ wild-type MCF-7 cells and in the right cornea an equal number of MK or PTN transfectant cells. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 3–4 days and capillaries progressed to reach the implanted pellet according to the scheme previously reported. The number of positive implants over the total implants performed was scored during each observation. The potency of angiogenic activity was evaluated on the basis of the number and growth rate of newly formed capillaries and an angiogenesis score was calculated [vessel density $\times$ distance from limbus] (15). A density of 1 corresponded to 0–25 vessels per cornea, 2 from 25 to 50, 3 from 50 to 75, 4 from 75 to 100, and 5 for more than 100 vessels. The distance from the limbus was graded with the aid of an ocular grid. Mouse xenograft experiments were performed essentially as described (16, 17). Cells in early log growth phase were scraped into PBSA and passed through a 25-gauge needle. After washing with PBSA, cells were resuspended in DMEM. MCF-7 cells ($1 \times 10^7$) mixed with an equal number of BALB/c 3T3 fibroblasts or MDA-435S cells in a total volume of 200 $\mu l$ of DMEM and injected unilaterally into the dorsal flank of $17\beta$-estradiol slow-release pellet- (1.5 mg of $17\beta$-estradiol/60-day release pellet) bearing mice. There were five mice in each experimental group. Perpendicular diameters of tumors were measured twice weekly with Vernier calipers. The tumor volume in mm$^3$ was calculated using the following formula (18): 

$$\text{Tumor volume} = \text{length} \times (\text{width})^2 \times 0.4.$$ 

Immunostaining of CD31 and BrdUrd. Frozen sections were incubated with rat anti-mouse CD31 (Ref. 19; MEC13) diluted 1:200 for 1 h. The sections were washed in PBS and incubated with swine anti-rat horseradish peroxidase (DAKO) diluted 1:100 for 30 min. Color development was performed with 3,3'-diaminobenzidine tablets (Sigma) according to the manufacturer’s instructions. The sections were washed and then incubated at 70°C in formaldehyde containing 0.317 $\times$ SSC for 40 min. The sections were washed in PBS and incubated with mouse anti-BrdUrd, Bu2Oa (DAKO), diluted 1:2 for 1 h, washed again, and then incubated with swine anti-rat horseradish peroxidase (1:100) for 30 min. The proliferating cells were visualized with the AEC substrate system (DAKO). The sections were counterstained with hematoxylin and mounted in apathies. Vascular density was determined by Chalkley counting (20).

RESULTS

Stable Transfection of MK and PTN into MCF-7 Breast Carcinoma Cells and Secretion of Active Protein. The full-length human PTN cDNA and mouse MK cDNA were cloned into pcDNA1neo, with expression under control of the cytomegalovirus promoter. Stable transfectants were isolated and designated MK, PTN, or neo (empty vector followed by the clone number). A MK or PTN mRNA transcript of the expected size was readily detectable in the transfected clones on hybridization to an appropriate human PTN or mouse MK probe (data not shown). Immunoblotting analysis showed the absence of PTN in neo cells but that human MK was present at a low level (Fig. 1). It is seen in Fig. 1 that high expression of human PTN and mouse MK was achieved in the transfected clones.

Addition of heparin affinity-purified MK or PTN to HUVECs stimulated growth when compared with heparin-purified neo cell-conditioned media (Fig. 2), confirming biological activity (21).

Expression of MK or PTN Has No Effect on MCF-7 Cell Growth In Vitro but Stimulates Tumor Formation In Vivo. The effect of MK and PTN expression on cell growth was then examined in vitro and in vivo. In vivo, we have shown that early passage MCF-7 cells require coimplantation with fibroblasts or other tumor lines for effective tumor take (16, 22). When coimplanted with 3T3 fibroblasts, a carcinoma, not a fibroma, grows and with MDA-435S cells a tumor of mixed hormone-dependent (MCF-7) and hormone-independent (MDA-435S) cells grows. In vitro, neither MK- nor PTN-transfected cell lines exhibited any difference in monolayer growth when compared with neo cells (Fig. 3). In contrast, when either MK- or PTN-transfected cells were s.c. implanted with an equal number of either BALB/c 3T3 fibroblasts or MDA-435S hormone-independent breast carcinoma cells into estrogen-supplemented nude mice, they gave rise to tumors that grew significantly faster than those from neo implants (Fig. 4). Thus, we conclude that despite the fact that expression of either MK or PTN confers no growth advantage on MCF-7 cells in monolayer culture, it does confer a growth advantage in vivo. Neither MK or PTN affected the estrogen requirement of the MCF-7 cells for growth.

MK and PTN Stimulate Endothelial Cell Proliferation and Vascular Growth In Vivo. Xenograft tumor sections were stained for the endothelial marker CD31 and the vascular density was determined by Chalkley counting (20). Tumors formed from the MK and PTN transfectants had a greater vascular density than those formed from neo cells (Fig. 5A). To examine the effect of MK and PTN on the proliferative index of the tumor vasculature, sections were immunostained with anti-CD31 and anti-BrdUrd. Fig. 5B shows representative sections from tumors originating from neo, MK-transfected, and PTN-transfected cells. Fig. 5C shows a higher tumor and endothelial cell proliferative index in the MK and PTN tumors compared with the neo tumors.

The vascular density of the MK and PTN tumors was largely uniform throughout the tumor. This is similar to tumors from MCF-7 cells that had been transfected with the angiogenic enzyme thyomidine phosphorlyase (22) but contrasts to VEGF-expressing tumors, which display areas of very high vessel density (vascular hot spots) while the background vascular density remains unchanged (16). Another unusual feature of the vessels in the MK and PTN tumors is that they appear very elongated within the section, in contrast to many tumor vessels, which display a tortuous corkscrew morphology (23).
Angiogenic Activity of MK and PTN Cells. In contrast to neo cells, both MK- and PTN-transfectant cell lines were found to be strongly angiogenic in the rabbit corneal assay (Fig. 6). The extent of the angiogenic response and the progression of the vascular front into the corneal stroma was similar for both the MK and PTN transfectants.

DISCUSSION

Despite extensive characterization of the MK and PTN genes and the regulation of their expression by retinoids and growth factors, the biological activities of these putative growth factors has remained controversial. MK has not previously been reported to have endothelial cell growth-stimulating or angiogenic activity, whereas the assertion that PTN is angiogenic has previously rested on the extrapolation of its ability to induce endothelial tube formation in vitro (24). Although endothelial cell tube formation in vitro most probably does correspond to a move toward the differentiated endothelial phenotype, there are many other molecular events that are essential components of in vivo angiogenesis.

Constitutive expression of either MK or PTN in MCF-7 breast carcinoma cells confers a growth advantage in vivo, without affecting
Fig. 5. Vascular density and proliferation within xenografted tumors. A, Quantitation of the vascular density of xenografted tumors by Chalkley counting after staining of CD31. Data represent mean vascular densities. Bars, SEM. B, Representative sections from frozen MDA-435S-containing xenografts were stained for CD31 (orange) and BrdUrd (red). Top, neo MCF-7; middle, PTN MCF-7; and bottom, MK MCF-7. C, Percentage of endothelial cell (top) and tumor cell (bottom) proliferation in the tumors. Bars, SD. mk, midkine; pin, pleiotrophin.

In vitro growth as a monolayer. In agreement with such findings, we have demonstrated both an increased vascular density (Fig. 5A) and an increased endothelial cell proliferative index (Fig. 5C) within MK and PTN tumors. These findings imply that an angiogenic activity could be responsible for the increased tumor growth. That MK and PTN are angiogenic was confirmed using the rabbit corneal assay (Fig. 6).

It is worthy of note that the effect of MK and PTN on HUVEC proliferation in vitro was quite small compared with that of acidic or basic fibroblast growth factor; however, VEGF is also a weak mitogen.
ROLE OF NEUROKINES MK AND PTN IN TUMORIGENESIS

Fig. 6. Angiogenic activity of the neo, MK, and PTN MCF-7 cell lines in the rabbit corneal assay. neo MCF-7; △ MK MCF-7; and ○ PTN MCF-7. Angiogenesis is expressed as an angiogenic score that was calculated as described in "Materials and Methods." Tabular data gives the number of positives of the five in each group. Two independent experiments gave similar results.

Fig. 6. Angiogenic activity of the neo, MK, and PTN MCF-7 cell lines in the rabbit corneal assay. neo MCF-7; △ MK MCF-7; and ○ PTN MCF-7. Angiogenesis is expressed as an angiogenic score that was calculated as described in "Materials and Methods." Tabular data gives the number of positives of the five in each group. Two independent experiments gave similar results.

for many endothelial cell lines, especially those of microvascular origin.\(^4\)

The vascular pattern in the MK- and PTN-expressing tumors was similar to those expressing the angiogenic enzyme thymidine phosphorylase but strikingly different from those expressing the smallest isoform of VEGF, VEGF\(_{121}\) (16, 22). MCF-7 cells expressing VEGF\(_{121}\) elicited an angiogenic response within 48 h but those expressing MK or PTN took 2 weeks. This is thought to be because the MK and PTN are sequestered on the cell surface by their heparin-binding activity, whereas VEGF\(_{121}\) is freely diffusible. VEGF\(_{121}\) expressing MCF-7 cells formed tumors with vascular hot spots very similar to those that have been shown to correlate with poor patient survival in primary human breast carcinomas (25). Whether or not vascular hot spots arise is possibly a result of how readily diffusible an angiogenic factor is. Thus, simple reaction-diffusion Turing models would predict that a freely diffusible angiogenic factor (such as VEGF\(_{121}\)) released into an inhibitory matrix would give rise to vascular hot spots, whereas if the factor remained bound to the extracellular surface (e.g., tight binding to heparin of MK and PTN) it would not (26).

It is unclear, as yet, whether the increase in vascular density arises solely from an increased rate of endothelial cell proliferation, or whether there is also a corresponding reduction in endothelial cell apoptosis. VEGF is known to be a survival factor for retinal endothelium (27).

The molecular receptors for MK and PTN have not yet been identified. PTN is known to bind to the membrane-extracellular protein syndecan-3 but appears not to signal on binding (28).

We have shown that overexpression of the neurokines PTN and MK leads to release of an endothelial growth-stimulating activity from the transfected cells, confers an angiogenic activity on the cells, and that this enhances both tumor growth and vascular density. These molecules therefore represent new targets for antiangiogenic cancer therapies.

\(^4\) Bicknell, unpublished data.

ACKNOWLEDGMENTS

We acknowledge the skilled assistance of Del Watling, Gary Saunders, and Sandra Peak in performing the xenograft experiments. R.C. thanks Dr. Stephen Fox (Department of Cellular Science, Oxford University, Oxford, United Kingdom) for showing her how to perform Chalkley counting. We are grateful to Dr. Rhys Jaggar (ICRF Angiogenesis Group) for helpful comments during the preparation of the manuscript.

REFERENCES

An Angiogenic Role for the Neurokines Midkine and Pleiotrophin in Tumorigenesis

Rangana Choudhuri, Hua-Tang Zhang, Sandra Donnini, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/9/1814

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/57/9/1814.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.