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PADMA-28, a traditional Tibetan herbal preparation, blocks cellular responses to bFGF and IGF-I

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Abstract—The growth factors basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-I) have been implicated in the pathophysiology of atherosclerosis and restenosis. The Tibetan herbal preparation PADMA-28 (a mixture of 22 plants which is used as an anti-atherosclerosis agent) was tested for its ability to inhibit the mitogenic activity of bFGF and IGF-I, growth factors involved in restenosis, atherosclerosis and tumour progression. DNA synthesis and proliferation of vascular smooth muscle cells, in response to serum bFGF, thrombin, or combinations thereof, were abrogated in the presence of microgram amounts of both the aqueous and organic, partially purified, extracts of PADMA-28. These fractions also inhibited IGF-I-mediated proliferation, migration and invasion of tumour cells responsive to IGF-I. The inhibition by PADMA 28 was reversible upon removal of the PADMA extracts, indicating that the effects were not related to cell toxicity. These and other properties (i.e., anti-oxidant activity) of PADMA-28 may be responsible for its beneficial effect as an anti-atherosclerotic agent, suggesting that this herbal preparation may have potential applications in the prevention of intimal hyperplasia and arterial stenosis secondary to coronary angioplasty and bypass surgery, as well as in the prevention and treatment of other vascular diseases and tumour growth and metastasis.

Key words: Atherosclerosis; bFGF; IGF-I; PADMA-28.

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1. INTRODUCTION

Endothelial cell injury plays a pivotal role in the deposition of fatty plaques in blood vessels, resulting in atherosclerosis, a phenomenon that can be categorized as a chronic inflammatory process (Libby *et al.*, 2002). Vascular endothelial cell injury or dysfunction results in complex multi-cellular interactions involving smooth muscle cells (SMC) and several host inflammatory cell types, including polymorphonuclear leucocytes, macrophages and platelets (Libby *et al.*, 2002). Inflammatory mediators trigger an overproduction of free radicals which promote peroxidation of lipids in LDL trapped in the sub-endothelial space. Products of LDL oxidation, as well as pro-inflammatory cytokines, induce endothelial cells to express cell-surface adhesion molecules and to secrete additional cytokines, chemotactic factors and growth factors. This leads to recruitment of monocytes and T-lymphocytes into the intima where the monocytes can differentiate into macrophages, the precursor of foam cells. In response to growth factors and cytokines, the SMC begin to proliferate in the intima, ultimately leading to narrowing of the lumen (Libby *et al.*, 2002; Ross, 1992, 1993).

Among the factors implicated in this process are interleukin-1 (Loppnow and Libby, 1990), platelet-derived growth factor (PDGF) (Ferns *et al.*, 1991; Jawien *et al.*, 1992), basic fibroblast growth factor (bFGF) (Edelman *et al.*, 1992; Linder *et al.*, 1991), heparin-binding epidermal growth factor (HB-EGF) (Raab *et al.*, 1997) and insulin-like growth factor 1 (IGF-I) (Balaram *et al.*, 1997; Bayes-Genis *et al.*, 2000). A potent growth-promoting activity towards SMC is also exerted by thrombin, which under certain conditions may be present within the vessel wall (Bar Shavit *et al.*, 1990; Schwartz, 1993).

These growth factors also promote intimal hyperplasia (restenosis), often induced by percutaneous transluminal coronary angioplasty (Libby *et al.*, 2002; MacBride *et al.*, 1998). Compounds which block the activity of one or more of these factors may, therefore, have preventative/therapeutic effects in the management of atherosclerosis and restenosis.

Two of the factors which appear to play a critical role in the progression of atherosclerosis and restenosis are bFGF and IGF-I. The FGFs are a family of at least 20 structurally related cationic polypeptides characterized by high affinity to heparin. They are highly mitogenic for vascular SMC and are among the most potent inducers of neovascularization (Vlodavsky and Christofori, 1998; Burgess *et al.*, 1989). Three of the cell types associated with the atherosclerosis process (endothelial cells, macrophages, SMC) synthesize bFGF (Raab *et al.*, 1997). Basic FGF may also accelerate the development of vasa vasorum from the adventitia, which provides nutrients to the atherosclerotic plaques (Edelman *et al.*, 1992).

IGF-I is a cationic 7.5-kDa peptide growth factor with a broad spectrum of biological activities (LeRoith *et al.*, 1992). It can mediate rapid and intermediate metabolic (insulin-like) processes, such as glucose uptake and glycogen synthesis, but its main function appears to be triggering long-term effects, including mitogenesis (Rubin and Baserga, 1995). In mediating its effect on DNA synthesis, IGF-I appears to act

in concert with so-called "competence factors" such as PDGF and bFGF (Cross and Dexter, 1991). In addition to its role in the control of cell proliferation, IGF-I has also been implicated in the regulation of cell motility and migration, biosynthesis of proteins and cell rescue from programmed cell death (Samani and Brodt, 2001).

Several lines of evidence suggest that IGF-I is implicated in atherosclerosis, namely (1) IGF-I is a known mitogen of SMC and a mitogen for endothelial cells, promoting endothelial cell tube formation. (2) Increased IGF-I receptor (IGF-IR) mRNA expression has been noted in atherosclerotic plaques, is localized to SMC and its expression may be mediated by low density lipoprotein (LDL). On the other hand, IGF-I has been found to increase macrophage uptake and degradation of LDL, suggesting a bi-directional regulation. (3) Modified LDL can augment IL-1 production in human peripheral blood mononuclear cells and IL-1 can in turn increase IGF-I production in endothelial cells. (4) IGF-I can modulate human platelet aggregation *via* extracellular calcium. Finally (5), IGF-I, but not bFGF, was identified as a potent survival factor for SMC (Ferns *et al.*, 1991; Hochberg *et al.*, 1992; Kamide *et al.*, 2000; Liu *et al.*, 2001; Loppnow *et al.*, 1990; Rifici *et al.*, 1994; Ruotolo *et al.*, 2000).

There is, however, also a common denominator between restenosis and tumour progression. Both processes involve poorly controlled cell proliferation, transmigration of cells *via* endothelial cells, invasion of adjacent tissues to cause metastases, and also cooperation among inflammatory cells and cytokines. The role played by bFGF and IGF-I in these processes might shed light on the aetiology of vascular diseases and tumour progression, and agent(s) that can modulate their functions might be utilized clinically.

One such modulating agent could be PADMA-28, a traditional Tibetan plant preparation. PADMA-28 is an herbal preparation based on an original Tibetan formulation comprising ingredients extracted from 22 defined plants and 2 minerals. It is produced commercially by PADMA AG and has been registered since 1977 by the Swiss IOCM (Intercantonal Office for the Control of Medicines). It is widely used in Switzerland and several other European countries to alleviate symptoms related to claudication and impaired peripheral circulation.

Aqueous preparations from these herbal mixtures have already been shown to function as potent anti-oxidants and anti-proteinases (Ginsburg *et al.*, 1999), as inhibitors of the generation of cytokines by human mononuclear cells stimulated by LPS and/or by lipoteichoic acid (Barak and Ginsburg, submitted), as well as suppressors of the development of diabetes in NOD mice (Weiss *et al.*, submitted). Also, several double-blind clinical studies have recently reported significant improvement in symptoms of peripheral atherosclerosis in PADMA-28 treated patients (Drabaek *et al.*, 1993; Ginsburg *et al.*, 1999; Sallon *et al.*, 1998).

In vitro studies suggest that this effect may be related to potent anti-oxidant activities of PADMA-28 on host immune functions (Matzner and Sallon, 1995). It modulates neutrophil functions (Matzner and Sallon, 1995) and improved fibrinolysis (Winther *et al.*, 1994).

In this communication we have attempted to establish whether this herbal extract could modulate cellular responses to bFGF and IGF-I in cultured SMC and lung carcinoma cells.

2. MATERIALS AND METHODS

2.1. Reagents

Recombinant human bFGF was kindly provided by Takeda Chemical Industries (Osaka, Japan). Human recombinant IGF-I was obtained from UBI (Lake Placid, NY, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI, foetal calf serum (FCS), penicillin, streptomycin and saline containing 0.05% trypsin, 0.01 M sodium phosphate (pH 7.4) and 0.02% EDTA (STV) were obtained from Biological Industries (Beit-Haemek, Israel). Tissue culture dishes were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA, USA). $Na^{125}I$ was obtained from Amersham (Amersham, UK). [^{3}H]Methyl thymidine (500 mCi/mmol) was obtained from New England Nuclear (Boston, MA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) and were of reagent grade.

Highly purified human α-thrombin was prepared from fraction III paste, evaluated for purity and characterized by Dr. J. W. Fenton II (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY, USA) (Bar Shavit *et al.*, 1990). Specific clotting activity of the α-thrombin preparation was 3975 U/mg. The preparation used in the experiments consisted of 98% α-, 1.8% β- and 0.7% γ-thrombin and was titrated to >85% active enzyme with *p*-nitrophenyl-*p*-guanidinobenzoate (NPGB). Poly(L-glutamic acid) (MW 5000–15 000) and poly(L-aspartic acid) (MW 5000–15 000) were used in the neutralisation experiments.

2.2. PADMA-28 extraction

PADMA-28 was supplied by PADMA AG (Zollikon Schwerzenbach, Switzerland), in a form of powder, the components of which are described in detail elsewhere (Matzner and Sallon, 1995; Sallon *et al.*, 1998). 100 mg amounts of PADMA-28 in 10 ml of normal saline (0.9% NaCl) were extracted with a mechanical vortex for 30 min at 37° C, centrifuged for 10 min at 10 000 rpm to remove insoluble material and the supernatants (approximately 1 mg/ml) were then stored at −20°C.

2.3. Smooth muscle cells (SMC)

SMC were isolated from bovine aortic media layer as previously described (Benezra *et al.*, 1994). Briefly, the abdominal segment of the aorta was removed, the fascia cleaned away under a dissecting microscope, the aorta cut longitudinally and small pieces of the media layer carefully stripped from the vessel wall. Two or three strips with average dimensions of 2–3 mm were placed in 100 mm tissue culture

dishes containing DMEM (4.5 g glucose/l), supplemented with 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin. Within 7–14 days, large patches of multi-layered cells migrated from the explants. The cells were sub-cultured into 100-mm^2 tissue culture plates ($(4–6) \times 10^5$ cells/plate) for approximately 1 week. Positive identification was on the basis of typical SMC morphology and immunopositivity for muscle form of actin as determined using a specific monoclonal antibody (HHF-35). This antibody does not recognize either endothelial cells or fibroblasts (Tsukada *et al.*, 1987). Clonal populations of bovine aortic endothelial cells (BAECs) were established and cultured in DMEM (1 g glucose/ litre) supplemented with 10% calf serum (CS). Recombinant human bFGF (1 ng/ml) was added every other day during the phase of active cell growth (Gospodarowicz *et al.*, 1976). Cells were cultured at 37°C in a 10% CO_2 humidified incubator, sub-cultured with 0.05% trypsin/0.02% EDTA and the experiments were performed with early (<20) cell passages.

Sparsely seeded vascular SMC were exposed to increasing concentrations of a saline extract of PADMA-28. At 2, 4 and 7 days after seeding, the cells were dissociated with trypsin/EDTA and counted.

The origin and properties of H-59 cells, a sub-line of Lewis lung carcinoma, were described in detail elsewhere (Long *et al.*, 1995). These cells were selected for the present study because of their well characterized multiple responses to IGF-I. They were maintained in RPMI medium supplemented with 10% FCS and antibiotics, as previously described (Long *et al.*, 1995, 1998).

2.4. [^{3}H]Thymidine incorporation assay

SMC were seeded at a confluent density in DMEM supplemented with 10% FCS into 24-well or 96-well culture plates. 24 h after seeding the medium was removed and replaced with new medium containing 0.2% FCS. 48 h later the cells were exposed to growth stimulants and [^{3}H]thymidine (1–5 μCi/well), with or without increasing concentrations of the saline extract or organic fractions of PADMA-28. DNA synthesis was assayed 24–48 h later by measuring the radioactivity incorporated into TCA-insoluble material (Benezra *et al.*, 1994). For cell counting, BAEC or SMC ($(2.5–5) \times 10^4$ cells/well) were seeded into 24-well culture plates and exposed to growth stimulants (bFGF, thrombin) and PADMA-28 extracts as described above. At various times after seeding, the cells were dissociated with trypsin/EDTA and counted in a Coulter Counter (Coulter Electronics) (Tsukada *et al.*, 1987). Each of the cell proliferation experiments described above was performed in triplicate at least three times and the variation between different experiments did not exceed $\pm$20%. H-59 cells, grown in RPMI containing 10% FCS, were washed twice with serum-free (SF) RPMI and cultured in SF-medium for 24 h. The cells were dispersed with PBS-EDTA and 2×10^3 cells/well seeded into 96-well polystyrene plates (Falcon) together with increasing concentrations of IGF-I, with or without PADMA-28 extracts, and incubated for 54 h. [^{3}H]Thymidine (1–5 μCi/well) was added for an additional 24 h, the cells lysed by repeated freezing and

thawing, and the cell lysates harvested onto paper filters using the Micromate 196 Harvester (Packard Instrument, Meriden, CT, USA). [^{3}H]Thymidine incorporation was monitored using the Beckman LS 8000 liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA).

2.5. Cell viability

To determine the effect of PADMA-28 on cell viability, 5×10^4 H-59 cells in 100 μl RPMI medium containing 0.2% BSA were incubated with or without PADMA-28 extracts for 48 h at 37°C in a 5% CO_2 atmosphere saturated with H_2O. Cell viability was then assessed with the MTT 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue) assay (Navab *et al.*, 2001). To each well, 10-μl quantities of MTT (Sigma) were added and the plates incubated at 37°C for 4 h. The plates were centrifuged for 5 min at low speed and 150–200 μl of the supernatant was removed from each well. To each well, 160 μl DMSO was added followed by mixing on a platform shaker. Absorbance was measured using an ELISA (3550 model Microplate reader, Bio-Rad, Richmond, CA, USA) at 540 nm.

2.6. RNA isolation and Northern blot analysis

RNA extraction and Northern blot analysis were performed as previously described (Long *et al.*, 1995; Navab *et al.*, 2001). RNA (30 μg of total RNA) was separated by electrophoresis on a 1.1% agarose gel containing 2 M formaldehyde and transferred to a nylon membrane (Hybond N, Amersham, Oakville, Canada) by capillary action. A 2.8-kb *Eco*RI restriction fragment of the human IGF-I receptor cDNA was isolated and used as a hybridisation probe. Hybridisation conditions were as previously described (Long *et al.*, 1995). The relative amounts of the mRNA transcripts were analysed by laser densitometry, using an LKB Bromma Ultroscan XL Enhanced Laser Densitometer and normalized relative to the internal 18S control.

2.7. IGF-I ligand-binding assay

The number of IGF-I binding sites on the tumour cells was analyzed as previously described (Navab *et al.*, 2001). Briefly, H-59 cells were cultured in RPMI-FCS in 24-well plates for 2–3 days. The culture medium was removed and replaced with SF-RPMI containing 1 mg/ml BSA. Binding assays were carried out 24 h later. To each well, SF-RPMI containing 8–1500 pM of ^{125}I-labelled IGF-I, 1 mg/ml BSA and 1 μg/ml leupeptin was added with or without graded concentrations of unlabelled IGF-I for 1 h incubation at 37°C. The cells were rinsed twice with ice-cold binding medium and solubilized in 0.01 M NaOH containing 0.1% Triton X-100 and 0.1% SDS. Cell number/well at the time of the assay was determined from triplicate control wells that were manipulated in the same manner. An aliquot from each well was removed and the radioactivity monitored in an LKB gamma counter.

The dissociation constant and the number of IGF-I binding sites were calculated using the Ligand program (Long *et al.*, 1995; Navab *et al.*, 2001).

3. RESULTS

3.1. A saline extract of PADMA-28 inhibits the proliferation of vascular SMC and endothelial cells

Figure 1A shows that, in the presence of 40 μg/ml PADMA-28, SMC proliferation was reduced by 40% and was further inhibited up to 80% in the presence of 60 μg/ml PADMA-28. In contrast, PADMA-28 had only a minor effect on the growth rate of BAECs (20%), even at a concentration of 60 μg/ml (Fig. 1B), indicating that vascular endothelial cells were less susceptible than vascular SMC to the inhibitory effect of PADMA-28.

3.2. Effect of polyanions on the mitogenic activity of bFGF

The possibility that the PADMA-28 effect on cell proliferation induced by bFGF might have been due simply to an electrostatic neutralization by polyanions abundantly present in PADMA-28 was investigated. The anionic nature of PAMA-28 was first established by mixing aqueous extracts of the herbal preparation with various amounts of the highly cationic poly(L-histidine) (PHSTD) at pH 6.8 (Ginsburg *et al.*, 1999). An immediate heavy opalescence occurred and the complexes formed between PHSTD and anionic ingredients in PADMA-28, were readily sedimented by centrifugation. This precipitated out most of the anti-oxidant capacities of PADMA-28 (data not shown). PHSTD also complexed with catalase, SOD and glucose oxidase, and facilitated their interaction with surfaces. We then tested the possible neutralization of the cationic growth factors either by the highly anionic poly(L-glutamic acid) (1–5 μg/ml) or with poly(L-aspartic acid) (1–5 μg/ml). The mixtures were added to SMC and [^{3}H]thymidine incorporation was determined. Neither polyanion, employed at approximately equimolar concentrations, inhibited the cell-proliferating activity of bFGF (data not shown).

3.3. PADMA-28 inhibits thrombin- and bFGF-induced SMC proliferation

Vascular SMC proliferation, in response to growth-promoting factors such as bFGF and thrombin, plays an important role in the pathogenesis of atherosclerosis (Libby *et al.*, 2002; Ross, 1993). The saline extract of PADMA-28 was tested for its ability to inhibit [^{3}H]thymidine incorporation induced by these growth factors. For this purpose, SMC were seeded at a low density (4×10^4 cells per 16 mm well), arrested at the G_0/G_1 phase by serum deprivation (0.2% FCS, 48 h) and incubated (48 h, 37°C) without or with 60 μg/ml PADMA-28 in the absence and presence of 7 nM thrombin, 1 ng/ml bFGF, or a combination of both. In the absence of PADMA-28, [^{3}H]thymidine incorporation was increased by 3-, 5- and 16-fold, respectively,

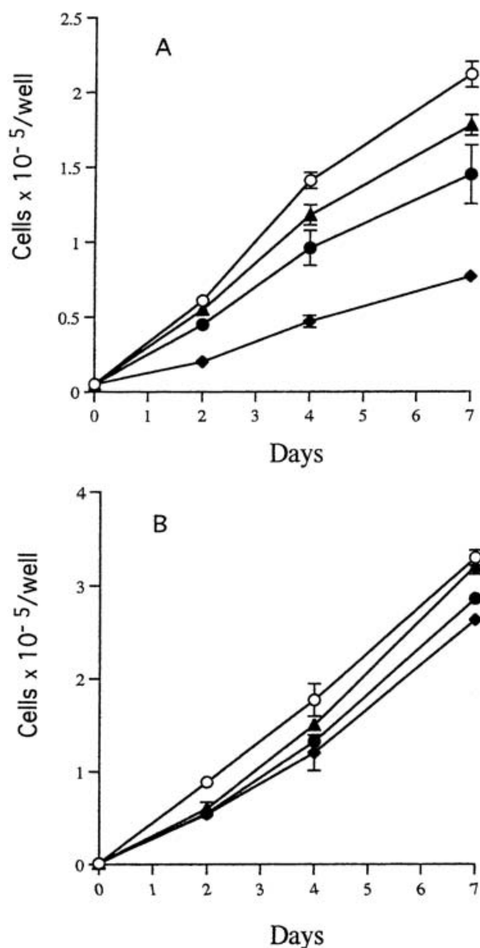

Figure 1. Effect of an aqueous extract of PADMA-28 on the proliferation of vascular smooth muscle and endothelial cells. Bovine aortic SMC (A) and bovine aortic endothelial cells (BAEC) (B) were seeded (1.5×10^4 cells/well) in complete medium into 24-well plates. 24 h afterwards, the cells were exposed to increasing concentrations of extracted PADMA-28 ((▲) 20 μg/ml, (●) 40 μg/ml, and (◆) 60 μg/ml). Control cells (○) were maintained in the absence of PADMA-28. On days 2, 4 and 7 after seeding, the cells were dissociated with STV and counted in a Coulter Counter. Each point represents the mean $\pm$ standard deviation (SD) from triplicate wells. All experiments were performed at least three times, and the variation between different experiments did not exceed $\pm$20.

indicating a profound synergistic effect of bFGF and thrombin (Fig. 2). PADMA-28 completely inhibited the growth promoting activity of bFGF and thrombin, as well as the synergistic stimulation of SMC proliferation induced by both bFGF and thrombin (Fig. 2). Even a higher inhibitory effect on cell proliferation was exerted by extracts obtained from PADMA-28 by organic solvents (not shown).

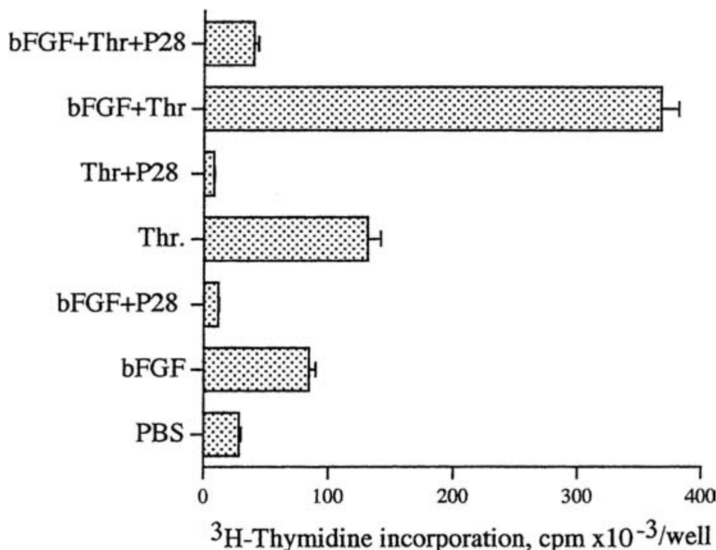

Figure 2. Inhibitory effect of saline-extracted PADMA-28 on the mitogenic activity of bFGF, thrombin or both on vascular SMC. Growth-arrested SMC were incubated in the absence or presence of 60 μg/ml saline-extracted PADMA-28 with 1 ng/ml bFGF, 7 μM thrombin, or both bFGF and thrombin for 48 h in the presence of [^{3}H]thymidine. Thymidine incorporation into TCA-insoluble material was determined as described in Materials and Methods. The background level of thymidine incorporation by growth arrested SMC ranged from 12 600 to 16 400 cpm in different plates. Each point represents the mean ± SD from triplicate wells.

3.4. PADMA-28 blocks IGF-I-induced DNA synthesis

To study the effect of PADMA-28 on cellular responses elicited by IGF-I, we used an established tumour cell line, H-59. This cell line was selected because the cells constitutively express functional IGF-I receptor levels and have well-characterized and reproducible responses to IGF-I (Long *et al.*, 1994, 1995, 1998).

The effect of PADMA-28 on H-59 cell viability was first assessed by the MTT assay following a 4–24 h incubation at 37°C with increasing concentrations of the saline extract. The results shown in Fig. 3 indicate that PADMA-28 had no significant cytotoxic effect on the cells at concentrations of 0.5–2.0 mg/ml. Reduction in cell viability was noted when the cells were incubated with concentrations of 3–5 mg/ml. Therefore, PADMA-28 was used in all subsequent experiments at concentrations not exceeding 2 mg/ml.

To test the effect of PADMA-28 on the mitogenic response to IGF-I, H-59 cells were incubated with different concentrations of IGF-I in the absence or presence of saline-extracted PADMA-28, and DNA synthesis was measured using the [^{3}H]thymidine incorporation assay. As shown in Fig. 4, there was a significant and concentration-dependent increase in [^{3}H]thymidine incorporation by untreated cells that peaked at 1 nM IGF-I (3.5-fold increase). At this IGF-I concentration, treatment with 1 mg/ml PADMA-28 reduced the response by 35%, while treatment

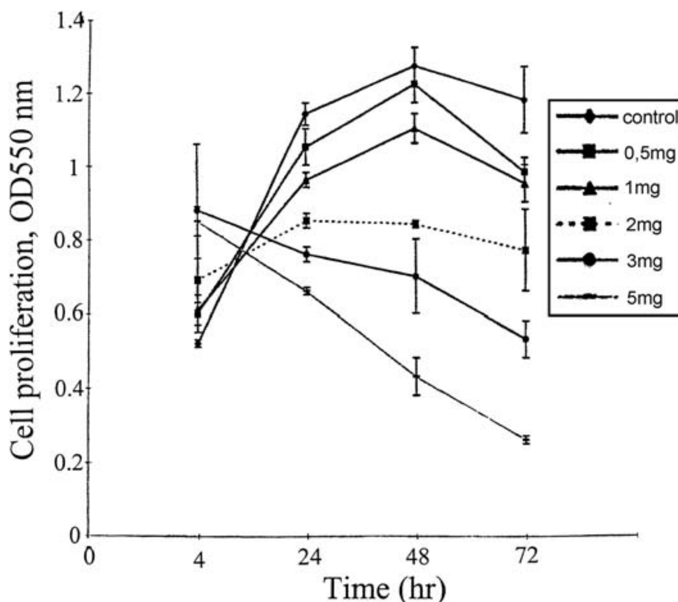

Figure 3. Effect of PADMA-28 on growth of H-59 cells. H-59 tumour cells were seeded into 96-well plates at a concentration of 5×10^4 cells/well and incubated for 4–72 h at 37°C with or without indicated concentrations of PADMA-28. MTT was added for an additional 4 h of incubation. Shown are results of a representative experiment carried out in triplicate. Bars denote SD.

with 2 mg/ml PADMA-28 completely abolished it. Similar to the observations with bFGF-induced SMC proliferation, we found that organic fractionation of PADMA-28 further enriched the inhibitory activity towards IGF-I-induced DNA synthesis (data not shown).

3.5. Effect of PADMA-28 on IGF-I binding affinity

Several mechanisms may account for the inhibitory effect of PADMA-28 on IGF-I, including a reduction in the number of cell surface receptors, reduced ligand binding affinity in the presence of PADMA-28, or both. To assess the effect of PADMA-28 on ligand binding and receptor expression, a ligand-binding assay was performed using ^{125}I-labelled IGF-I in the presence of excess concentrations of unlabelled IGF-I. Scatchard analysis of the binding results revealed that in the presence of 2 mg/ml PADMA-28, the binding affinity of IGF-I ($K_d = 6.61$ nM) was significantly reduced relative to control, untreated H-59 cells ($K_d = 2.68$ nM). However, in these cells, the number of IGF-I binding sites as calculated from the B_{max} values was not significantly altered (Fig. 5).

3.6. Effect of PADMA-28 on IGF-I receptor mRNA synthesis

To determine whether PADMA-28 affected IGF-I receptor mRNA synthesis, a Northern blot analysis was performed on H-59 cells incubated in the absence or

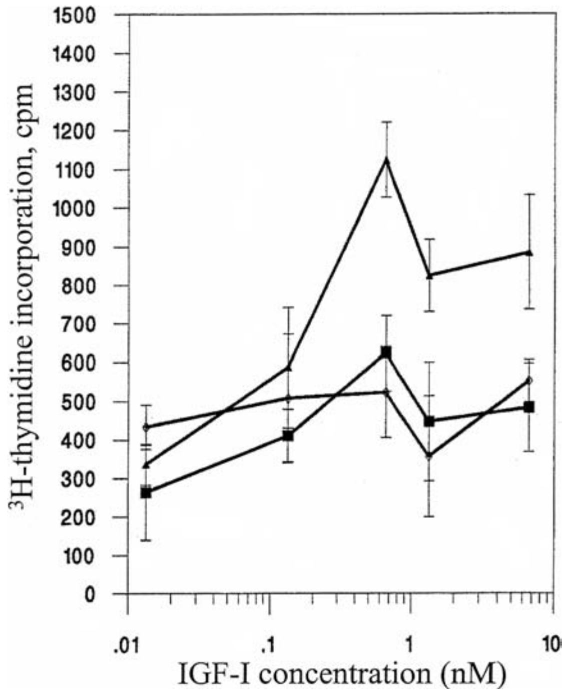

Figure 4. PADMA-28 inhibits the proliferative mitogenic response of H-59 cells to IGF-I. Serum-starved cells were seeded in 96-well microtiter plates and incubated with the indicated concentrations of IGF-I in the absence (▲) or presence of 1 mg/ml (■) or 2 mg/ml (◇) PADMA-28. The results are means of triplicates and are expressed as the increase in [^{3}H]thymidine incorporation by cells relative to control unstimulated cells.

presence of PADMA-28 for 48 h. The results shown in Fig. 6 indicate that PADMA-28 reduced the levels of both the 11-kb and 7.6-kb IGF-IR transcripts. At a concentration of 2 mg/ml, PADMA-28 reduced the levels of these transcripts by 40 and 22%, respectively.

4. DISCUSSION

The present study was undertaken in an effort to elucidate the biological basis for the reported beneficial effects of PADMA-28 in the treatment of atherosclerosis and intermittent claudication. Both bFGF and IGF-I have been implicated in the ontogeny of atherosclerosis, which is considered to be a distinct inflammatory process. These involve complex synergistic interactions among oxidants, growth factors, proteinases, cationic peptides, PLA2 and cytokines (Ginsburg *et al.*, 1999).

Therefore, we reasoned that PADMA-28, which had already been shown to possess potent anti-oxidant, anti-proteinase (Ginsburg *et al.*, 1999) and anti-cytokine properties (Barak and Ginsburg, submitted), might perhaps also inhibit the activity of growth factors shown to be directly involved in SMC proliferation,

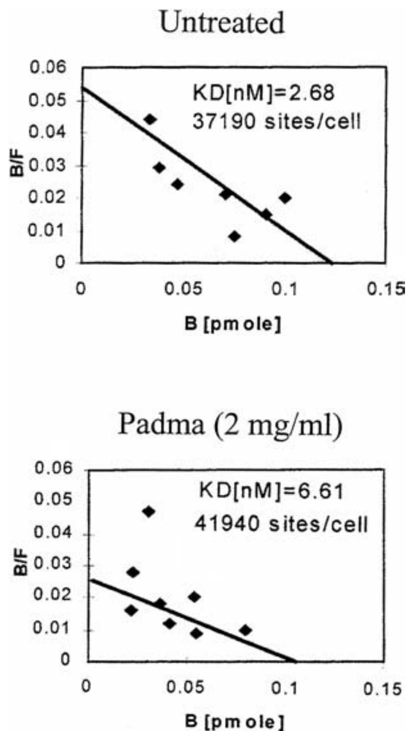

Figure 5. Scatchard analysis of IGF-I binding in the presence of PADMA-28. [^{125}I]IGF-I at concentrations of 8–1500 pM was added to H-59 cell monolayers with or without graded concentrations of unlabelled IGF-I and the cells were incubated for 1 h at 37°C. The dissociation constant and the number of IGF-I binding sites for each cell line were calculated using the Ligand program.

causing restenosis, a process which is often observed in patients undergoing balloon angioplasty.

Using cell systems, our results show that aqueous extracts of PADMA-28 do, indeed, block cellular responses to these cationic peptide growth factors. This activity could be further enriched by organic extraction of PADMA-28 combined with chromatography and fractionation (unpublished results).

However, it is obvious that, since PADMA-28 is comprised of 22 different herbs, each containing a large number of still mostly unidentified compounds (Ginsburg *et al.*, 1999), it will be of great importance to isolate and characterize those agents, or combinations thereof, which are directly involved in their anti-proliferative and anti-tumourigenic activities.

Cationic peptide growth-promoting factors are involved in cell proliferation and migration associated with intimal hyperplasia in response to endothelial cell injury, as well as with tumour progression. These growth factors can be generated by endothelial cells (EC), SMC, tumour cells or cells in adjacent tissues. Figure 1 shows that saline extracts of PADMA-28 significantly reduced the proliferation

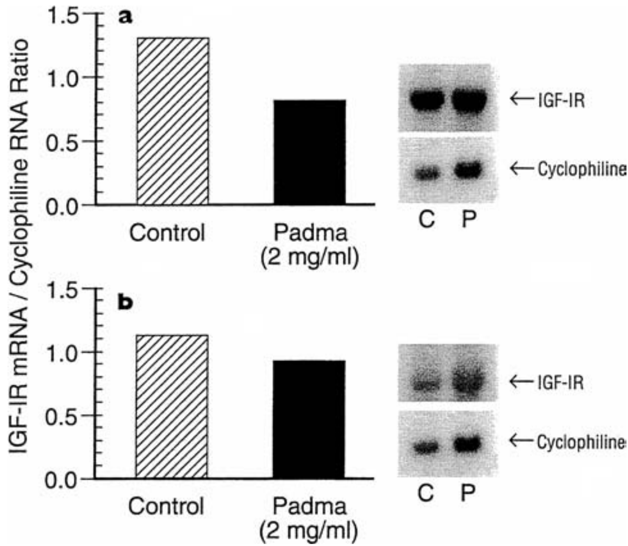

Figure 6. Northern blot analysis of IGF-IR mRNA expression in PADMA-28-treated H-59 cells. 30 μg of total RNA from H-59 cells was size-fractionated by electrophoresis on 1.1% formaldehyde–agarose gels. The blots were probed first with a ^{32}P-labelled IGF-IR cDNA and then with a ^{32}P-labelled oligonucleotide probe for cyclophiline. Laser densitometry was used to measure the intensity of the bands, which is expressed relative to control bands of cyclophilin mRNA. Shown are the results for the 11-kb IGF-IR transcript (a) and for the 7.6-kb transcript (b).

of SMC, but to a lesser extent that of EC, in a concentration-dependent manner, regardless of whether or not SMC proliferation was induced by serum, bFGF, alpha thrombin or a combination of bFGF and thrombin.

The finding that PADMA-28 markedly inhibited the growth promoting effect of both bFGF and thrombin (Fig. 2) emphasizes that the selective inhibition of SMC proliferation might be an advantage in repair of the vessel wall involved in the pathophysiology of atherosclerosis.

The findings that, at non-cytotoxic concentrations, PADMA-28 also had the capacity to suppress the mitogenic response of H-59 tumour cells, suggests a potential application of this herbal preparation in suppressing tumour growth.

It is also important to note that bFGF and IGF-I are stored in the extracellular matrix and in the stromal compartment of the vessel wall and primary tumours, and can be released by proteolytic and glycosidic enzymes (Medalion *et al.*, 1997; Vlodavsky *et al.*, 1991, 1993). In this respect, PADMA-28 has been shown to inhibit neutrophil-derived elastase activity (Ginsburg *et al.*, 1999) and tumour-derived heparanase (unpublished results).

We have demonstrated that, in order to exert inhibition of cell proliferation induced by the highly cationic bFGF, PADMA-28 had to be present in the cell culture medium. However, it had to be ruled out that PADMA, rich in polyanions, could simply neutralize the highly cationic growth factor. Premixing the cationic bFGF with the highly anionic peptides, polyglutamate or polyaspartate, to mimic the

highly anionic nature of the PADMA-28 preparation, failed to diminish its induction of cell proliferation. This indicates, therefore, that charge effects *per se* were not the cause for the inhibitory activity of PADMA and that other mechanisms of action should be considered.

It is conceivable that some of the components of crude PADMA-28, as well as the enriched fractions (data not shown) could either bind to the receptor, or to the ligand by non-electrostatic forces to modulate its affinity. They could also modify the interaction between IGF-I and one of the IGF-I binding proteins produced by H-59 cells, thereby reducing the binding affinity to the receptor (Clemmons *et al.*, 1993).

This assumption had been strengthened by the findings (Fig. 5) that 2 mg of PADMA-28 extract significantly reduced the binding affinity of IGF-I, but had no effect on the numbers of binding sites.

The data in Fig. 6 showing that, although PADMA-28 caused a reduction of IGF-IR mRNA synthesis levels, there was no apparent reduction in the numbers of IGF-I binding sites, is also of note. The reason for this apparent discrepancy, however, is not clear. It might be possible that post-transcriptional mechanisms, which may not be affected by PADMA-28, or might even be enhanced by it, are possibly involved and may compensate for the reduction in mRNA levels. It is also possible that the decrease in mRNA levels which was observed following a 48-h incubation with PADMA-28, was secondary to an earlier reduction in IGF-I binding affinity observed at 24 h and the result of a feed-back mechanism where IGF-IR synthesis is regulated by the level of IGF-I binding.

Another mechanism of action of PADMA-28 is by inhibiting the activity of enzymes involved in the release of ECM-resident growth factors. The fact that both aqueous and methanol extracts (data not shown) of PADMA-28 efficiently inhibited not only neutrophil elastase (Ginsburg *et al.*, 1999), but also tumour cell heparanase (unpublished results), might be of great significance. Heparanase was shown to be involved in tumour-cell metastasis and angiogenesis, and in the release of active bFGF and other heparin-binding growth factors from the basement membrane and extracellular matrix where they are stored as a complex with heparin sulfate (Elkin *et al.*, 2001; Medalion *et al.*, 1997; Vlodovsky *et al.*, 1991, 1993; Vlodavasky and Christofori, 1998).

Our results show that PADMA-28 had a profound effect on the ability of tumour cells to respond to IGF-I. This effect of PADMA-28 on the IGF-IR/IGF-I system was manifested at several levels. While PADMA-28 had a minor effect on the synthesis of IGF-IR mRNA (Fig. 6), it significantly reduced the binding affinity of IGF-I to its receptor and inhibited tumour cell proliferation in response to IGF-I. Moreover, it reduced tumour cell motility (not shown). Thus, PADMA-28 had an inhibitory effect on several critical determinants of the malignant phenotype.

The findings presented may shed some light on other observations reported with PADMA-28. The observed reduction in IGF-I responsiveness in the presence of PADMA-28 may also explain the finding that PADMA-28 inhibited the proliferation

of SMC and to a lesser extent of endothelial cells in response to bFGF. IGF-I is mitogenic for SMC, but induces mainly cell migration and tube formation in endothelial cells (Kamide *et al.*, 2000; Liu *et al.*, 2001). Since bFGF is a competence factor which requires progression factors, such as IGF-I, to commit cells into mitosis, it is conceivable that the inhibitory effect of PADMA-28 on SMC proliferation resulted, at least in part, from the inhibition of IGF-I binding, an effect not demonstrable in endothelial cells. These results may also be relevant to the protective effect exerted by PADMA-28 on plaque formation in atherosclerosis, a process driven by IGF-I, and on LDL synthesis, where IGF-I is also implicated.

Taken together, our results identify growth-factor-induced cellular proliferation as a target of PADMA-28 and also provide a rationale for further purification and characterization of the active constituent(s). In view of the critical role that bFGF and IGF-I play in the induction of several pathological processes including atherosclerosis and tumour growth and metastasis, our results provide a compelling rational for further examination of the therapeutic potential of PADMA-28. Identification of the active constituents in PADMA-28 may have clinical implications for the prevention of restenosis, atherosclerosis, tumour spread and of additional vascular disorders.

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