Influence of protamine on adhesion, chemotaxis and proliferation of human vascular smooth muscle cells

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Summary It has been shown that, in streptozotocin diabetic rats, protamine-retarded insulin administered in vivo stimulates intimal hyperplasia in balloon-injured carotid artery. The aim of this study was to evaluate the influence of protamine on cultured human vascular smooth muscle cells (hVSMC), by observing its effects on adhesion, chemotaxis and proliferation. hVSMC were isolated during abdominal surgery, cultured and utilized at passages 6–10. We observed that protamine stimulates: 1) cell adhesion in the concentration range 0.04–20 µg/ml (analysis of variance, ANOVA, $p < 0.0001$); 2) cell chemotaxis in the absence of fetal calf serum (FCS) in the concentration range 1–200 µg/ml (ANOVA, $p < 0.0001$) and in the presence of 1% FCS in the concentration range 5–200 µg/ml (ANOVA, $p < 0.0001$), further enhancing the chemotaxis induced by 10% FCS in the concentration range 20–200 µg/ml (ANOVA, $p < 0.0001$); 3) cell proliferation and ³H-thymidine incorporation from 1 to 5 µg/ml (ANOVA, $p < 0.0001$); 4) cell c-fos oncoprotein

nuclear expression. We also observed that protamine effects on chemotaxis, proliferation and c-fos expression are inhibited by heparin that human insulin stimulates cell proliferation and ³H-thymidine incorporation (ANOVA, $p < 0.0001$) at concentrations equal to or greater than 480 pmol/l and that these effects of insulin persist in the presence of protamine. In conclusion, protamine influences hVSMC behaviour by interfering with biological functions involved in atherogenesis. The concentrations used in this short-term in vitro study were higher than those probably occurring in vivo in patients chronically treated by protamine-retarded insulin preparations: further studies, therefore, are needed to evaluate the safety of protamine as a retardant of insulin action in vivo. [Diabetologia (1997) 40: 67–75]

Keywords Protamine, insulin, heparin, vascular smooth muscle cells, diabetic angiopathy, atherosclerosis.

Protamine-retarded insulins are widely used in clinical practice. However, little information is available

Received: 23 May 1996 and in revised form: 19 September 1996

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Abbreviations: hVSMC, Human vascular smooth muscle cells; MEM, minimum essential medium; FCS, fetal calf serum; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid; PBS, phosphate buffered-saline; ANOVA, analysis of variance.

on the fate of subcutaneously injected protamine. It is known that 30–50% of patients treated with protamine produce specific anti-protamine antibodies belonging to the IgG or IgE classes [1], which are responsible for the 40–50-fold increased risk of potentially lethal allergic reactions to intravenous protamine given to antagonize the anticoagulant effect of heparin during cardiovascular procedures [2]. Further studies should be undertaken to determine the amount of protamine metabolized at the injection site during chronic subcutaneous administration and the amount which enters the bloodstream, the site of in vivo protamine catabolism and the effect of the interaction between protamine and its specific

antibodies on protamine localization at the vascular or kidney level. Without this information, it is difficult to predict the vascular concentrations of protamine in patients who have been treated daily with protamine-retarded insulin for a long time period. Since the long-acting (18–36 h) protamine-zinc insulin preparation contains about 0.3 mg protamine per mg insulin and the intermediate-acting (12–24 h) neutral protamine Hagedorn (NPH) preparation contains about 0.1 mg protamine per mg insulin, one could probably foresee a plasma concentration in the ng/ml range after injection of an insulin-protamine preparation. It is more difficult to foresee the levels attained after a period of chronic treatment, for the reasons mentioned. The lack of interest in the fate of injected protamine reflects the common belief that it is only a retardant of insulin action.

Protamine is a basic, polycationic protein rich in arginine, whose major physiological role is to condense the DNA contained in mature spermatozoa in an insoluble complex [3]. It is used not only to retard insulin action, but also to neutralize the anticoagulant effect of therapeutically administered heparin, an anionic glycosaminoglycan with which it interacts in a 1 : 1 ratio through its cationic sites [4]. The ability of protamine to antagonize the heparin effects should be carefully considered since heparin not only has anticoagulant properties. Actually, endogenous heparin or heparin-like substances play an important role in maintaining the vascular smooth muscle cells (VSMC) in a quiescent growth state [5]. Heparin binds to VSMC and is internalized via a receptor-mediated endocytosis [6, 7]; within the cells, it exerts important effects on growth-related events, inhibiting both early G0/G1 transition [8] as well as mid-to-late G1 progression [9]. Furthermore, it selectively inhibits a protein kinase C-dependent mitogenic signalling pathway, including a repression of the proto-oncogene *c-fos* induced by phorbol esters [10]; finally, it displaces mitogens, such as fibroblast growth factor, from the arterial wall [11]. Glycosaminoglycan deficiency is involved in the pathogenesis of atherosclerosis [12] and glomerular hyperpermeability in diabetes [13, 14]; furthermore, administration of glycosaminoglycans, heparin or heparin-like substances, prevents [15] or reduces [16] the glomerular hyperpermeability of diabetic nephropathy. The fact that protamine inhibits (through its cationic sites) the effects exerted by heparin (through its anionic sites) raises therapeutic concerns, since the lack of anionic charges is considered one of the most relevant pathogenetic factors of diabetic angiopathy [12–14].

It has been impressively shown by Edelman et al. [17], using a model of balloon catheter injury in rats, that i) a continuous release of protamine ($33\text{--}821\text{ }\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) from the subcutaneous tissue for 1 week before and 2 weeks after arterial injury increases intimal hyperplasia and cell proliferation and

antagonizes the heparin-induced inhibition of intimal growth; ii) exposure of rat aortic VSMC to $1\text{--}10\text{ }\mu\text{g}/\text{ml}$ protamine enhances cell proliferation and inhibits the anti-proliferative effect of heparin; iii) in streptozotocin diabetic rats, neointimal hyperplasia after carotid injury, which is dramatically lower than in control animals, is increased by the administration of crystalline insulin ($40\text{ U} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) slightly, but not significantly, whereas protamine-zinc insulin administration ($20\text{ U} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) increases neointimal hyperplasia 5.2-fold; the effects on cell proliferation, however, do not linearly correspond to those on vascular injury exacerbation, suggesting a role for protamine in VSMC migration. In summary, in rats protamine alone or protamine-insulin preparations induce proliferative responses of VSMC that are typical of the atherosclerotic process [18]. It should be considered that the doses of protamine/zinc insulin used in the rats were much higher than those usually administered in humans (20 vs $0.6\text{ U} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ maximum); the protamine doses were also much higher (about 300 vs $8\text{ }\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ maximum).

This important study in rats compelled us to evaluate the effects of protamine on human VSMC (hVSMC). In particular, we aimed to investigate a) whether protamine influences not only hVSMC proliferation, but also *c-fos* oncoprotein expression, adhesion and chemotaxis; b) whether the protamine effects on hVSMC are antagonized by heparin.

Furthermore, protamine has been described to inhibit platelet-derived growth factor receptor activity [19] and the mitogenic properties of the extracellular matrix and fibroblast growth factor [20], whereas it potentiates those of epidermal growth factor [20]. These effects are interesting per se: for instance, probably owing to its ability to inhibit some growth factors involved in angiogenesis, protamine shows anti-angiogenic properties [21]. Insulin is able to stimulate VSMC proliferation [22]; it is not known, however, whether protamine interferes with this property of insulin. In this study, therefore, we aimed to investigate whether protamine enhances, reduces or simply does not affect the proliferative activity of insulin.

Materials and methods

Culture of hVSMC. Microarterioles for isolation of hVSMC were obtained from a single non-diabetic, normotensive, heart disease free patient undergoing abdominal surgery. The cells for culture were prepared as described and shown to accumulate acetylated low density lipoproteins, and to stain positively with smooth muscle α -actin antibody, but negatively with Factor VIII antibody [23]. These cells have previously been shown in our laboratory to adhere and proliferate on fibronection in adhesion and proliferation assays [24] and to produce endothelin [25]. The cells were grown in Minimum Essential Medium (MEM) supplemented with 10 mmol/l L-glutamine, 10 mmol/l (N-tris [hydroxymethyl]-methyl-2-aminoethane-

sulphonic acid (TES)/4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES), 100 U/ml penicillin and streptomycin, 1 mmol/l sodium pyruvate and 10% fetal calf serum (FCS), all from Boehringer Mannheim (Mannheim, Germany). Confluent cells were passaged by trypsinization and were utilized at passages 6–10 for the experimental procedures.

Adhesion assay with hVSMC. The assay was performed as described by Bourdon and Ruoslahti [26]. Ninety-six-well polystyrene plates (Sterilin Limited, Hounslow, UK) were coated with protamine (Salmon Protamine, Sigma, St. Louis, Mo., USA) by dissolving the protein (concentration range 0.04–20 µg/ml) in distilled water and adding 50 µl of the protein solution per well. The plates were then dried overnight under a sterile hood and blocked with MEM plus 0.25% bovine serum albumin (BSA) for 1 h at 37°C. Subconfluent monolayer cultures were harvested by treatment with Trypsin/EDTA/EGTA (0.05/0.1/0.01%), washed once with MEM plus 0.25% BSA and the cells were resuspended in MEM plus 0.1% BSA at 5×10^5 /ml. One hundred microlitres of cell suspension was added to the coated wells and incubated for 2 h to allow adhesion of cells. At the end of incubation, the wells were washed three times with 0.2 ml/well of phosphate buffered saline (PBS), the adherent cells were fixed to the substratum using 3.5% formaldehyde in PBS for 10 min and stained with 0.5% toluidine blue in PBS. The cells were lysed with 100 µl/well 1% sodium dodecyl-sulphate and the optical density read at 620 nm in the Elisa reader. The intra-assay coefficient of variation for the adhesion assay was less than 5% and the inter-assay coefficient of variation was less than 10% at the concentrations tested. In the experiments where the dose-response curve for the adhesion was evaluated, protamine at each concentration ranging from 40 ng/ml to 20 µg/ml was tested in six wells per plate; four plates were used per experiment, and the experiments were repeated three times.

To show the appearance of hVSMC when plated on protamine, pictures of the cells that had been allowed to adhere for 2 h were taken with an inverted microscope at 100× magnification. As a negative control, pictures of hVSMC that had been allowed to adhere for 2 h in uncoated and blocked wells were taken.

To evaluate an eventual ability of insulin as a coating agent, the hormone (Recombinant Human Insulin, Calbiochem, La Jolla, Calif., USA) was prepared at concentrations ranging from 240 pmol/l to 15.72 nmol/l and 50 µl were added per well; subsequently the plates were treated as previously described.

Chemotaxis assay. The assay was conducted in 48-well chemotaxis chambers (Neuroprobe, Bethesda, Md., USA). Each well consists of a lower part in which the solutions of chemoattractants are placed, and of an upper part where the cell suspensions are placed. The lower and upper parts are separated by polycarbonate filters with 8-µm pores coated with Type 1 collagen at 10 µg/ml. In a first set of experiments, the cell suspensions were prepared at 5×10^5 /ml in MEM plus 0.1% BSA and protamine was added to MEM plus 0.1% BSA, at concentrations ranging from 1 to 200 µg/ml. In a second set of experiments, designed to evaluate whether protamine is able to increase chemotaxis of hVSMC in the presence of a low serum concentration, both the chemotactic solution and the cell suspension were prepared in MEM plus 1% FCS and protamine was added to the chemotactic solution at concentrations ranging from 5 to 200 µg/ml. In a third set of experiments, designed to evaluate whether protamine is able to increase chemotaxis induced by serum, the chemotactic solution was prepared with MEM plus 10% FCS and protamine was added at concentrations ranging from 20 to 200 µg/ml, while the cells

were suspended in MEM plus 0.1% BSA. As negative control for chemoattractants MEM plus 0.1% BSA was used, while as positive control MEM plus 10% FCS was used; in both cases the cells were suspended in MEM plus 0.1% BSA. To evaluate the inhibitory effect of heparin on chemotaxis induced by protamine, heparin (Eparina Vister, sodium salt, 150 IU/mg; Parke-Davis, Barcelona, Spain) at 200 µg/ml was added to the chemotactic solution containing protamine at 200 µg/ml.

To evaluate the eventual effect of insulin on chemotaxis, the hormone was added to the chemotactic solution, both with and without 1% or 10% FCS, at concentrations ranging from 240 pmol/l to 15.72 nmol/l, either alone or in addition to protamine at the concentration of 10 and 200 µg/ml.

After assembling, the chambers were incubated at 37°C in 5% CO₂ for 8 h. Finally the chambers were disassembled, the filters were fixed in methanol and stained with Harris' haematoxylin and eosin. The cells in the upper part of the filter were removed, while the cells that had migrated to the lower part of the filter were counted. Four 400× fields per well were counted, three wells were tested in each experiment per each concentration and the experiments were repeated four times. In our laboratory, intra-assay coefficient of variation for the chemotaxis assay was less than 10%, while the inter-assay coefficient of variation was less than 20% at the concentrations tested.

Proliferation assays. Proliferation of hVSMC was tested by counting the number of cells and by evaluating thymidine incorporation.

Cell count. The cell proliferation assay was conducted in 24-well plates by seeding 10^4 hVSMC per well in MEM plus 10% FCS. After 24 h, the medium was changed to MEM plus 1% FCS and maintained for 48 h. At the end of this period, the MEM plus 1% FCS was replaced with the same medium and the various substances were added at the specified concentrations: protamine from 1 to 5 µg/ml, heparin at 5 and 10 µg/ml, insulin from 60 to 1920 pmol/l, protamine at 5 µg/ml plus heparin at 5 or 10 µg/ml, protamine at 1 and 2 µg/ml plus insulin from 240 to 1920 pmol/l. After 48 h the medium was replaced together with the additions just described. After an additional overnight incubation, the cells were trypsinized and the number of cells per well was counted in a haemocytometer, performing two counts per well. Each concentration was tested in quadruplicate and the experiments were repeated three times. Proliferation experiments employing cell counting showed an intra-assay coefficient of variation less than 5% and an inter-assay coefficient less than 10%.

Thymidine incorporation assay. The thymidine incorporation assay was conducted in 24-well plates by seeding 10^4 hVSMC per well in MEM plus 10% FCS. After 24 h, the medium was changed to MEM plus 1% FCS and maintained for 48 h. At the end of this period, the MEM plus 1% FCS was replaced with the same medium and the various substances were added at the same concentrations specified for the proliferation assay: protamine from 1 to 5 µg/ml, heparin at 5 and 10 µg/ml, insulin from 60 to 1920 pmol/l, protamine at 5 µg/ml plus heparin at 5 or 10 µg/ml, protamine at 1 and 2 µg/ml plus insulin from 240 to 1920 pmol/l. After 48 h the MEM plus 1% FCS was replaced and the various test substances and tritiated thymidine (methyl-³H-thymidine, 20 Ci/mmol; Du Pont de Nemours Italiana, Milan, Italy) at 1 µCi/ml were added. Following a further 15-h incubation, the medium was removed, the wells were washed with 2 ml Hank's Balanced Salt Solution, then with 2 ml of 10% ice-cold trichloroacetic acid for 10 min. After two additional 5-min washes with trichloroacetic acid, the cell monolayers were dissolved with 500 µl/well 1% sodium dodecyl-

sulphate in 0.3 mol sodium hydroxide and the radioactivity evaluated in a beta counter (Packard Instrument B. V., Groningen, The Netherlands). Each concentration was tested in quadruplicate and the experiments were repeated three times. Proliferation experiments employing thymidine incorporation showed an intra-assay coefficient of variation less than 5% and an inter-assay coefficient less than 10%.

Immunofluorescence staining. In order to evaluate the effect of protamine on c-fos oncogene expression, the cells were incubated with MEM plus 1% FCS for 48 h on cover-glasses in six-well plates. After this period, the medium was replaced with MEM plus 1% FCS and various test substances added: protamine at 5 $\mu\text{g/ml}$, protamine at 5 $\mu\text{g/ml}$ plus heparin at 20 $\mu\text{g/ml}$. The incubation was maintained for 1, 2, 6, and 24 h. Finally, the medium was removed, the wells were washed with PBS, the cells were fixed with acetone-methanol (9:1) at -20°C , rehydrated with PBS, and finally stained with a sheep antibody to c-fos oncoprotein (Cambridge Research Biochemicals, Wilmington, Del., USA) followed by a fluorescein isothiocyanate-conjugated rabbit antibody to sheep IgG (Sigma, St. Louis, Mo., USA). The coverslips were then mounted on a slide and observed in a fluorescence microscope. Each slide was read independently by three researchers and a semiquantitative score was assigned to each slide.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical evaluation was performed by means of analysis of variance (ANOVA); pairwise comparisons were evaluated by means of Fisher's least significant difference [27]. Statistical calculations were elaborated utilizing the program StatView 4.01 (Abacus Concepts Inc., Berkeley, Calif., USA) for the Macintosh computer.

Results

Adhesion. Protamine promoted the attachment of hVSMC to the substrate and induced the formation of a cell monolayer (Fig. 1A); in the absence of a coating agent no cells attached to the polystyrene plates (Fig. 1B). The phenomenon of adhesion to protamine showed a dose dependency (Fig. 2) ($p < 0.0001$): the effect of protamine was detected starting from 40 ng/ml coating concentration. Insulin at physiological or supraphysiological concentrations ranging from 240 pmol/l to 15.72 nmol/l did not induce adhesion of hVSMC ($p = \text{NS}$).

Chemotaxis. Protamine by itself stimulated chemotaxis of hVSMC (Fig. 3): the effect was present when protamine was incubated in the absence of FCS at concentrations from 1 to 200 $\mu\text{g/ml}$ ($p < 0.0001$) (Fig. 3A). The effect of protamine at concentrations ranging from 5 to 200 $\mu\text{g/ml}$ was also detected when the assay was conducted in the presence of 1% FCS added both to the cell suspension and to the chemoattractant solution ($p < 0.0001$) (Fig. 3B). When protamine was added to the chemoattractant solution together with 10% FCS, it enhanced the chemotactic effect of 10%

Fig 1. A. hVSMC adhering to protamine at 1 $\mu\text{g/ml}$; cells appear as a homogeneous monolayer with an enlarged cytoplasm. **B.** In the absence of protamine, the cells do not adhere to the wells, do not expand their cytoplasm and form aggregates. Photographs taken in an inverted microscope at $100\times$ magnification

FCS at concentrations ranging from 20 to 200 $\mu\text{g/ml}$ ($p < 0.0001$) (Fig. 3C). The chemotactic effect of protamine alone, of protamine in the presence of 1% FCS or the stimulating effect of protamine on chemotaxis induced by 10% FCS were inhibited by heparin at equivalent concentrations: Figure 3 shows the results for protamine 200 $\mu\text{g/ml}$ and heparin 200 $\mu\text{g/ml}$.

Insulin at concentrations ranging from 240 pmol/l to 15.72 nmol/l did not affect chemotaxis in the experimental conditions examined (without FCS, with 1% and with 10% FCS) ($p = \text{NS}$); furthermore, chemotaxis induced by protamine was absolutely unchanged in the presence of insulin, even at the highest concentration tested ($p = \text{NS}$).

Proliferation. Protamine dose-dependently stimulated cell proliferation, evaluated by cell counting, starting from 1 up to 5 $\mu\text{g/ml}$ ($p < 0.0001$) (Fig. 4A). Protamine dose-dependently stimulated the incorporation of tritiated thymidine by hVSMC from 1 to 5 $\mu\text{g/ml}$ ($p < 0.0001$) (Fig. 4B).

Heparin alone inhibited cell proliferation evaluated by counting the number of cells ($p = 0.008$) (Fig. 4A) and measuring thymidine incorporation ($p = 0.0001$) (Fig. 4B). The stimulating effect of protamine at 5 $\mu\text{g/ml}$ on cell number (Fig. 4A) and thymidine incorporation was inhibited by heparin at 5 and 10 $\mu\text{g/ml}$ (Fig. 4B) ($p < 0.0001$ both for cell number and thymidine incorporation). When hVSMC were incubated with protamine at 5 $\mu\text{g/ml}$ plus heparin at

Fig. 2. Effect of protamine on hVSMC adhesion. Protamine dose-dependently stimulates the adhesion of hVSMC (ANOVA: $p < 0.0001$)

5 µg/ml or with protamine at 5 µg/ml plus heparin at 10 µg/ml, the cell proliferation was higher than when the cells were incubated with heparin alone both by cell counting (at 5 µg/ml, $p = 0.0045$, and at 10 µg/ml, $p = 0.0369$, respectively) (Fig. 4 A) and by thymidine incorporation (at 5 µg/ml, $p = 0.0024$, and at 10 µg/ml, $p = 0.022$, respectively) (Fig. 4 B).

Insulin itself induced a slight but significant proliferation of hVSMC as evaluated by cell counting ($p < 0.0001$) (Fig. 5 A) and thymidine incorporation ($p < 0.0001$) (Fig. 5 B). The effect on cell counts and on thymidine incorporation was significant starting from 480 pmol/l.

When insulin at 240, 480, 960 and 1920 pmol/l was added to protamine at 1 µg/ml, its effects on hVSMC counts and on thymidine incorporation were significant ($p = 0.0013$ and $p = 0.0007$, respectively) (Fig. 6). Similar effects were obtained with protamine 2 µg/ml ($p = 0.0179$ and $p = 0.0024$, respectively).

Immunofluorescence staining for c-fos. hVSMC maintained in 1% FCS and stained for c-fos oncoprotein showed weak nuclear staining (Table 1). Cells incubated with protamine at 5 µg/ml showed strong nuclear staining for the c-fos oncoprotein after the first two hours of incubation; later, the staining became weaker. When protamine at 5 µg/ml was incubated in the presence of heparin at 20 µg/ml, the nuclei of hVSMC did not stain for c-fos oncoprotein.

Discussion

The present study shows that: 1) protamine induces the adhesion of hVSMC in the absence of FCS. 2) Protamine acts as a chemotactic agent for hVSMC, both in the absence and in the presence of FCS, and enhances the chemotaxis induced by 10% FCS. 3) Protamine stimulates the proliferation of hVSMC, as shown by cell counting and by thymidine

Fig. 3. (A–C) Effect of protamine on hVSMC chemotaxis. **A.** Protamine dose-dependently stimulates the chemotaxis of hVSMC starting from 1 µg/ml. (ANOVA: $p < 0.0001$). The chemotactic effect of protamine at 200 µg/ml is inhibited by heparin at 200 µg/ml (⊗) added to the chemotactic solution. **B.** Protamine dose-dependently stimulates the chemotaxis of hVSMC in the presence of 1% fetal calf serum added both to chemotactic solution and to cell suspension starting from 5 µg/ml (ANOVA: $p < 0.0001$). The chemotactic effect of protamine at 200 µg/ml is inhibited by heparin at 200 µg/ml (⊗) added to the chemotactic solution. **C.** Protamine dose-dependently stimulates the chemotaxis induced by 10% fetal calf serum starting from 20 µg/ml (ANOVA: $p < 0.0001$). The effect of protamine at 200 µg/ml is inhibited by heparin at 200 µg/ml (⊗) added to the chemotactic solution. P + H, Protamine at 200 µg/ml + Heparin at 200 µg/ml

incorporation, and induces c-fos oncoprotein expression. 4) Heparin inhibits the hVSMC proliferation induced by FCS, the chemotactic and proliferative effects of protamine, and the protamine ability to activate c-fos oncoprotein expression. 5) At very high physiological (~480 pmol/l) and supraphysiological (≥ 960 pmol/l) concentrations, insulin slightly stimulates hVSMC proliferation and thymidine incorporation, whereas, even at highly supraphysiological concentrations, it does not affect hVSMC adhesion or chemotaxis. 6) In the presence of protamine, the effects of insulin on hVSMC proliferation remain significant.

Fig. 4. (A, B) Effect of protamine and heparin on hVSMC proliferation. **A.** Protamine dose-dependently increases hVSMC counts (ANOVA: $p < 0.0001$). Heparin inhibits basal hVSMC counts (ANOVA: $p = 0.008$). The stimulating effect of protamine at 5 $\mu\text{g}/\text{ml}$ on hVSMC counts is inhibited by heparin at 5 and 10 $\mu\text{g}/\text{ml}$ ($p < 0.0001$). When hVSMC are incubated with protamine 5 $\mu\text{g}/\text{ml}$ + heparin 5 $\mu\text{g}/\text{ml}$, or with protamine 5 $\mu\text{g}/\text{ml}$ + heparin 10 $\mu\text{g}/\text{ml}$, the number of cells that proliferate is higher than when the cells are incubated with heparin alone at 5 $\mu\text{g}/\text{ml}$ ($p = 0.0045$) or at 10 $\mu\text{g}/\text{ml}$ ($p = 0.0369$), respectively. **B.** Protamine dose-dependently stimulates thymidine incorporation by hVSMC (ANOVA: $p < 0.0001$). Heparin inhibits basal incorporation of tritiated thymidine by hVSMC (ANOVA: $p = 0.0001$). The effect is significant at 5 and at 10 $\mu\text{g}/\text{ml}$ ($p = 0.039$ and $p = 0.0027$, respectively). The stimulating effect of protamine at 5 $\mu\text{g}/\text{ml}$ on tritiated thymidine incorporation in hVSMC is inhibited by heparin at 5 and 10 $\mu\text{g}/\text{ml}$ ($p = 0.0001$). When hVSMC are incubated with protamine at 5 $\mu\text{g}/\text{ml}$ + heparin at 5 $\mu\text{g}/\text{ml}$, or with protamine at 5 $\mu\text{g}/\text{ml}$ + heparin at 10 $\mu\text{g}/\text{ml}$, tritiated thymidine incorporation is higher than in the presence of heparin alone at 5 $\mu\text{g}/\text{ml}$ ($p = 0.0024$) or at 10 $\mu\text{g}/\text{ml}$ ($p = 0.022$), respectively. C, Control; P, protamine ($\mu\text{g}/\text{ml}$); H, heparin ($\mu\text{g}/\text{ml}$)

As far as we know, this is the first study to evaluate the effects of protamine on hVSMC. We employed hVSMC taken from arterioles, and not from aorta or its main branches, which are the vascular segments more prone to atherosclerosis, only because it is very difficult to obtain specimens of these arteries from non-atherosclerotic subjects, and we aimed to study

Fig. 5. (A, B) Effect of insulin on hVSMC proliferation. **A.** Insulin increases hVSMC counts (ANOVA: $p < 0.0001$). When each concentration is evaluated independently, the effect is significant at 480 pmol/l ($p = 0.024$), 960 pmol/l ($p < 0.0001$) and 1920 pmol/l ($p < 0.0001$). **B.** Insulin stimulates thymidine incorporation by hVSMC (ANOVA: $p < 0.0001$). When each concentration is evaluated independently, the effect of insulin is significant at 480 pmol/l ($p = 0.05$) and increases with increasing insulin concentrations: at 960 pmol/l, $p = 0.0007$, at 1920 pmol/l, $p < 0.0001$

cells not affected by pathological changes. We cannot exclude, however, that cells from large arteries would show a different sensitivity to the proliferative effects of protamine or insulin.

The sensitivity of our cells to protamine is very similar to that of rat aortic cells [17] as far as proliferation is concerned, since protamine concentrations equator greater than 1 $\mu\text{g}/\text{ml}$ are required.

As far as we know, this is the first study taking into account the direct protamine effects on VSMC adhesion and chemotaxis; therefore, we are not able to compare the protamine doses we used with those of other studies. The protamine concentrations capable of increasing hVSMC adhesion are relatively low, in the ng/ml range, and this fact could have some relevance for effects in patients treated with protamine-retarded insulin.

Protamine concentrations utilized for the chemotaxis assay seem to be elevated when compared to those

Fig. 6. (A, B) Effect of insulin + protamine on hVSMC proliferation. **A.** When insulin at 240, 480, 960 and 1920 pmol/l is added to protamine at 1 µg/ml, the increase of hVSMC count induced by insulin is significant (ANOVA: $p = 0.0013$). **B.** When insulin at 240, 480, 960 and 1920 pmol/l is added to protamine at 1 µg/ml, the increase of thymidine incorporation induced by insulin is significant (ANOVA: $p = 0.0007$). C, Control; P, protamine (µg/ml); I, insulin (pmol/l)

active on adhesion and proliferation; however, it has been observed that the concentrations of chemotactic substances present on the upper surface of the filters and able to activate chemotaxis are as low as one-fifth of those added to the lower chamber [28]: thus, the concentrations of protamine active on chemotaxis are similar to those that induce adhesion and proliferation.

This study shows that protamine is able to modify cultured hVSMC chemotaxis and proliferation features in acute experiments at concentrations that are probably higher than those attained in vivo, even if specific studies are needed to define the therapeutic protamine concentrations reached in chronically treated patients.

The second part of the discussion considers the possible mechanisms involved in the protamine actions we observed.

The process of adhesion is necessary in VSMC, as in other anchorage-dependent cells, for normal cell functions, i.e. the ability to structure the cytoskeleton, to alkalize intracellular pH, to contract in response to

Table 1. Effect of protamine and protamine plus heparin on nuclear expression of c-fos oncoprotein by hVSMC evaluated by indirect immunofluorescence

Incubation time (h):	1	2	6	24
Fetal calf serum 1 %	+/-	+/-	+/-	+/-
Protamine 5 µg/ml	+ /++	++ /+++	++	+ /++
Protamine 5 µg/ml + Heparin 20 µg/ml	+/-	-	-	-

mediators like angiotensin II, to migrate, and finally, to proliferate in response to mitogenic stimuli [29]. In the present study we showed that in vitro protamine directly interacts with hVSMC, allowing adhesion to the substrate at nanomolar concentrations suggesting a high affinity of protamine for the cell surface. This effect of protamine is not surprising, since other cationic molecules, such as polylysine, are able to increase cell adhesion; for instance, packed human erythrocytes are attached to polylysine-coated polyacrylamide beads so firmly that the further lysis of attached cells leaves the beads covered by plasma membranes [30]. Actually, the high positive charges of polycationic molecules facilitate the interaction with the negative charges of the cell surfaces and of extracellular matrix. Protamine itself has been used to interact with the anionic charges of glomerular basement membranes, a specialized type of extracellular matrix [31]. Other cationic proteins also interact with the glomerular wall when injected in vivo [32, 33].

As far as chemotaxis is concerned, we showed that protamine stimulates hVSMC chemotaxis both with and without serum. VSMC migration from the media to the intima in response to chemotactic substances, such as platelet-derived growth factor and other factors derived from platelets and macrophages, plays a key role in the formation of the atherosclerotic plaque [18]. Protamine could take part in this process both through a direct activation of chemotaxis and/or interplay with the effects of other agents. For instance, protamine has been shown to interfere in different ways with the receptors of different growth factors [19, 20]. However, the dose-response effect of protamine-induced chemotaxis up to the huge dose of 200 µg/ml in the absence of FCS, suggests an active role for protamine on its own in VSMC chemotaxis.

As far as hVSMC proliferation is concerned, we observed that protamine stimulates both the increase of cell number and the incorporation of tritiated thymidine. Furthermore, we demonstrated for the first time that protamine stimulates c-fos oncoprotein expression. The effect on hVSMC proliferation is present at a low FCS concentration (1%), which is enough to allow the survival of the cells, but minimizes the effect of serum itself.

Protamine presents different mechanisms by which it could affect VSMC proliferation: 1) it

interacts with growth factor receptors, which are glycoproteins containing negatively charged carbohydrate moieties, decreasing the action of some growth factors and increasing that of others [19, 20]; 2) it reduces the well-known antiproliferative effects of heparin or heparin-like substances [17], the mechanisms of which have already been extensively described in the introduction; 3) it could also modulate cell proliferation on its own.

VSMC produce heparin-like compounds that inhibit their proliferation [34]; in our study, however, the dose-response relationship of the protamine effects on cell proliferation even when high doses are reached does not exclude the presence of an active role for protamine on its own on VSMC proliferation.

The reciprocal interactions between heparin and protamine, on the other hand, are particularly interesting, as pointed out in the introduction. We have already mentioned that heparin specifically represses *c-fos* mRNA induced by phorbol-esters, but not by epidermal growth factor, suggesting that it selectively inhibits a protein kinase C-dependent mitogenic signaling pathway [10]. The heparin effect on *c-fos* is closely associated with its growth-inhibitory activity, since heparin does not suppress *c-fos* mRNA induction in cells derived from a strain of rats resistant to its antiproliferative effects [10]. Our observation that heparin suppresses the *c-fos* oncoprotein expression activated by protamine in hVSMC could be due to an effect of heparin on this proto-oncogene, or to the fact that heparin counteracts the interaction of protamine with the cells by means of a charge mechanism, and, consequently, blocks any protamine-mediated intracellular event.

In this study, we also evaluated the effects of insulin on hVSMC. Insulin has been previously described as a potentially atherogenic hormone since it not only stimulates VSMC proliferation, but also activates the Na^+/H^+ antiporter, increases VLDL synthesis and deposition in the vessel wall and the cholesterol transport in the VSMC, and enhances the sympathetic tone [35]. On the other hand, insulin, through nitric oxide, exerts beneficial vasodilating [36–38] and platelet anti-aggregating [39, 40] properties, that are lost in the hyperinsulinaemic, insulin-resistant states [41, 42], inducing us to speculate that the resistance to beneficial vascular effects of insulin could also play a role in the pathogenesis of arterial hypertension and atherosclerosis. An open question is whether the proliferative effects described in cultured VSMC *in vitro* are reproducible in man when physiological insulin concentrations are attained *in vivo*, where they could be attenuated by nitric oxide-dependent mechanisms mediated by other cell types, such as endothelial cells and platelets. In our study, insulin was only able to stimulate hVSMC proliferation from the concentration of 480 pmol/l, i.e., in the very high postprandial range. On the other hand, even at

very high, non-physiological concentrations (see 15.72 nmol/l), it was unable to stimulate adhesion and chemotaxis of hVSMC. When protamine, at active concentrations, was added to insulin, it showed additive effects. It is very important to note that in our experiments the relative concentrations of insulin and protamine are different on a per weight basis than those usually employed in insulin/protamine preparations, protamine being present in much higher doses. Therefore, our results do not support the conclusion that protamine increases the proliferative effects of insulin in the clinical setting. The meaning of these experiments is that protamine, which reduces the effects of some growth factors and increases those of others [19, 20], neither reduces nor potentiates the proliferative effects of insulin.

The present study, carried out as a short-term experiment *in vitro* in cultured hVSMC, does not prove that protamine is atherogenic at clinical doses as a retardant of insulin preparations: actually, we evidenced proliferative effects starting from 1 µg/ml, whereas the circulating range of protamine concentrations reached *in vivo* should be of ng/ml at maximum. Our study, however, supports the conclusion that protamine, commonly used as a retardant of insulin action, should not be considered inert or neutral from the vascular point of view.

It is known that endogenous heparin-like substances play a pivotal role in protection against vasculopathy *in vivo* [5], and that their lack in diabetic patients contributes to the pathogenesis of angiopathy [12–14]: for these reasons, our study does not exclude that protamine concentrations reached *in vivo* after daily administration, for years and decades, could antagonize the protective actions of endogenous heparin-like molecules, inhibiting the already deficient vascular repair provided by these substances in patients at high risk of developing atherosclerosis.

In conclusion, this work could stimulate further research aiming to elucidate whether protamine is actually able to influence the evolution of diabetic angiopathy.

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