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Protamine Sulfate Inhibits Mitogenic Activities of the Extracellular Matrix and Fibroblast Growth Factor, but Potentiates That of Epidermal Growth Factor

G. NEUFELD* AND D. GOSPODAROWICZ
Cancer Research Institute, University of California San Francisco, California 94143

Protamine sulfate, an inhibitor of angiogenesis in vivo, markedly inhibits the ability of angiogenic factors such as acidic or basic fibroblast growth factor (aFGF, bFGF) to stimulate the proliferation in vitro of either BHK-21 cells or vascular endothelial cells. The inhibition is reversible, and cells remain viable even after prolonged exposure to protamine sulfate. Protamine sulfate inhibits the mitogenic effects of both growth factors by preventing them from binding to their common cell surface receptors. It also inhibits the mitogenic activity of the extracellular matrix produced by bovine corneal endothelial cells. This substrate has been shown in previous studies to replace the requirement for FGF of many cell types. In contrast, protamine sulfate potentiates the mitogenic activity of epidermal growth factor (EGF). This indicates that protamine sulfate also acts at cellular sites which are not associated with FGF receptors.

Basic and acidic fibroblast growth factor (bFGF, aFGF) are two closely related mitogens that have a 55% structural homology (Esch et al., 1985) and interact with the same classes of receptors present on both established cell lines or normal diploid cells (Neufeld and Gospodarowicz 1985, 1986; Neufeld et al., 1987; Olwin and Hauschka, 1986). Both growth factors are mitogenic for a wide variety of neuroectodermal and mesenchymal cells, in particular, capillary and large vessel derived endothelial cells (Gospodarowicz, 1985; Gospodarowicz et al., 1978) and both are angiogenic in vivo (Gospodarowicz et al., 1986b).

Previous studies have shown that protamine sulfate, a small basic protein that binds to DNA is anti-angiogenic in vivo (Taylor and Folkman, 1982; Majewski et al., 1984). The molecular basis of this activity is presently unknown. In the present study, we have analyzed whether protamine sulfate could inhibit the bioactivity of FGF as well as that of the extracellular matrix (ECM) produced by bovine corneal endothelial cells, which has been shown in previous studies to mimic the effect of FGF on both cell proliferation and differentiation (Gospodarowicz, 1984).

MATERIALS AND METHODS

Materials

Protamine, protamine sulfate, histone, thymidine, gelatin, and transferrin were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbeco's modified Eagle's medium-H16 (DMEM) and Ham's F-12 medium were obtained from Grand Island Biological Co. (Grand Island, NY). Calf serum (CS) was obtained from Hy Clone (Sterile Systems Inc., Logan, UT). Tissue culture dishes were from Falcon Plastics (Oxnard, CA), gentamicin from Schering Co. (Kenilworth, NJ), and fungizone from E.R. Squibb & Sons (Princeton, NJ). ^{3}H-Thymidine (18.2 Ci/mmole) was from New England Nuclear (Boston, MA), and ^{125}I was from Amersham (Chicago, IL).

Bovine pituitary FGF (basic FGF) was purified as previously described (Gospodarowicz, 1986) using heparin-Sepharose affinity chromatography. Acidic FGF was purified from brain using the same procedure (Böhlen et al., 1985). Growth factor homogeneity was determined by SDS-PAGE, by amino acid composition, and by sequence analysis of the amino terminal portion of the molecules (Gospodarowicz et al., 1985; Böhlen et al., 1985). Epidermal growth factor (EGF) was purified as described (Savage and Cohen, 1972).

Human high density lipoproteins (HDL, $1.07 < d < 1.21$ g/cm^3) were obtained from human plasma by differential ultracentrifugal flotation (Gospodarowicz et al., 1984).

Coating of plates with gelatin or ECM

To coat the plates with gelatin, a 2-ml solution of 0.2% gelatin in PBS was added to 35-mm tissue culture plates. Plates were allowed to sit overnight at 4°C. The next day, the solution was removed, and plates were washed with PBS (Neufeld et al., 1986). Plates coated with an ECM produced by bovine corneal endothelial cells were prepared as described previously (Gospodarowicz, 1984).

Cell cultures and growth studies

A baby hamster kidney cell line (BHK-21) was used (Neufeld et al., 1986). Also used were vascular endothelial cells derived from bovine aortic arch (ABAE) (Gos-

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*To whom reprint requests/correspondence should be addressed.

podarowicz et al., 1986a). BHK-21 cells were cultured in a 1:1 (v/v) mixture of DMEM and Ham's F-12 media (DF medium) supplemented with 5% CS, 50 μg/ml gentamicin, and 0.25 μg/ml fungizone. Stock plates of BHK-21 cells grown in the presence of serum-supplemented medium were washed once with PBS and then exposed (2–3 min, 24°C) to a solution containing 0.9% NaCl, 0.01 M sodium phosphate (pH 7.4), 0.05% trypsin, 0.02% EDTA (STV solution, Difco Laboratories Inc., Detroit, MI). When cells rounded up, they were suspended in DF medium supplemented with 2 mg/ml BSA and then spun down. The cell pellet was resuspended in DF medium. An aliquot of the cell suspension was then counted in a Coulter counter (Coulter Electronic, Inc., Hialeah, FL), and cells were distributed at the initial cell density, as indicated in the figure legends.

When cultures were to be grown in serum-free medium, seeding of the cultures was done on gelatinized dishes (Neufeld et al., 1986) in the presence of 2 ml of DF medium supplemented with 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and with the various factors being analyzed for their ability to support cell growth. Insulin (5 μg/ml) and transferrin (5 μg/ml) were added only once. Protamine, histone, FGF, or EGF were added every other day. Cultures were kept in a humidified incubator (37°C) in a 5% CO_2/95% air environment.

ABAE cells were routinely cultured in DMEM supplemented with 10% CS, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, and 1 ng/ml basic FGF (Gospodarowicz et al., 1986a). Stock plates of ABAE cells were trypsinized as described for BHK-21 cells. ABAE cells were resuspended in medium supplemented with 2 mg/ml crystalline BSA and then spun down. The cell pellet was resuspended in DMEM, and aliquots containing the appropriate cell number were then seeded onto gelatin- or ECM-coated dishes containing 2 ml of DMEM supplemented with 50 μg/ml gentamicin, 0.25 μg/ml fungizone, 10 μg/ml transferrin, and 750 μg/ml HDL (protein/ml) (serum-free medium). Transferrin and HDL were added only once. FGF and protamine or histone were added after the cells plated at the concentrations indicated in the figure legends. Further additions were made every other day. Cultures were kept in a humidified incubator (37°C) in a 10% CO_2/95% air environment.

Cell growth measurement

For cell growth measurements, cells were seeded, as described above on 35-mm plastic dishes coated or not with gelatin or ECM. After 6 hr, triplicate plates were trypsinized and cells counted to determine plating efficiency. At appropriate times, triplicate plates were trypsinized. Cell number was determined using a Coulter counter. The morphological appearance of the cultures was observed by phase contrast microscopy.

^{3}H-Thymidine incorporation

BHK-21 cells (5×10^4) were seeded in DF medium supplemented with insulin and transferrin onto 35-mm ECM-coated dishes. After the cells plated, 1 μCi/ml ^{3}H-thymidine together with 18 ng/ml thymidine was added to the cultures in a 10-μl aliquot of medium. After a 24-hr incubation at 37°C, the medium was removed, and the cultures were washed twice with 2 ml of cold PBS.

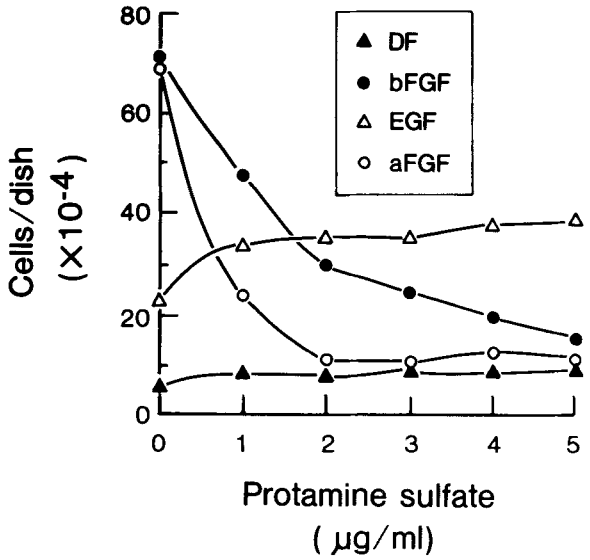

Fig. 1. Protamine sulfate inhibits the mitogenic activity of bFGF and aFGF but stimulates EGF activity. BHK-21 cells (4×10^4) were seeded in 35-mm gelatinized dishes containing 2 ml of DF medium supplemented with transferrin and insulin. Following the plating of the cells, protamine sulfate and the various growth factors were added to the desired concentrations. The growth factors were: aFGF (10 ng/ml), bFGF (1 ng/ml), EGF (5 ng/ml), or no growth factor added (DF). Protamine sulfate and the various growth factors were added every other day. The cultures were trypsinized on day 4 and cells counted in a Coulter counter.

0.1 N NaOH (1 ml) was then added and a 0.5-ml aliquot was counted in a Beckman scintillation counter, model LS-8,000.

Binding and covalent cross-linking of ^{125}I-bFGF to its BHK-21 cell surface receptor

Iodination of bFGF and cross-linking of ^{125}I-bFGF to BHK-21 cell surface receptors were done as previously described (Neufeld and Gospodarowicz, 1985). Binding of ^{125}I-bFGF (250,000 cpm/ng) to BHK-21 cells was done essentially as previously described (Neufeld and Gospodarowicz, 1985), with the following modifications: cells were grown to confluence in 96-well plates. The volume of binding buffer was 100 μl/well. At the end of the binding, each well was washed three times with 200 μl of wash buffer (1 mg/ml BSA in PBS). The cells were then lysed with solubilization buffer containing 2% Nonidet P-40 and 1 mg/ml BSA in PBS, and aliquots were counted in a Beckman gamma counter model 5500.

RESULTS

Effect of protamine sulfate on the proliferation of BHK-21 cells exposed or not to aFGF, bFGF, and EGF

BHK-21 cells exposed to DF medium supplemented with insulin and transferrin do not proliferate unless exposed to growth factors such as aFGF, bFGF, or EGF (Fig. 1). In order to analyze the effect of protamine sulfate on the response of the cells to these various mitogens, low density BHK-21 cell cultures maintained in

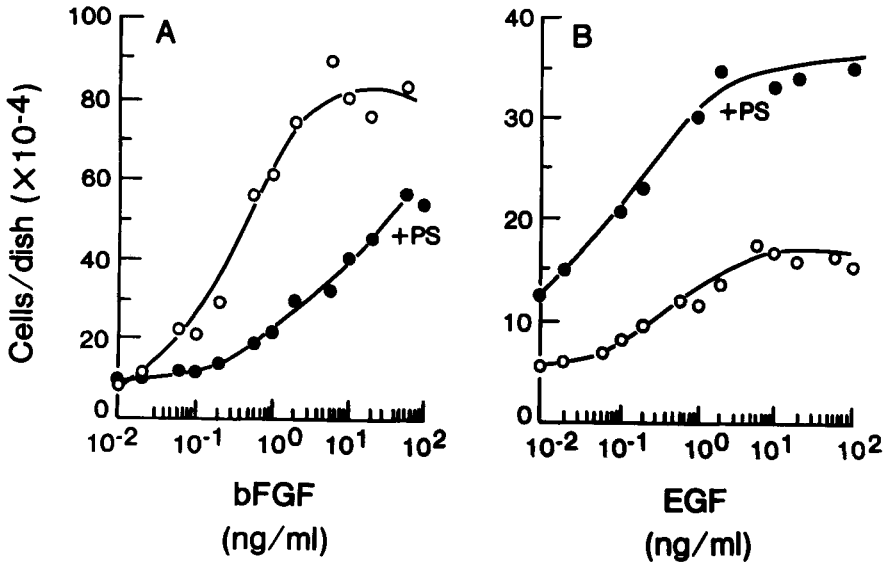

Fig. 2. Log-dose response curve of EGF and bFGF in presence or absence of protamine sulfate. BHK-21 cells (4×10^4) were seeded in 35-mm gelatinized dishes containing 2 ml of DF medium suplemented with transferrin and insulin. Following the plating of the cells, each of the various dishes received (●) or not (○) 5 μg/ml protamine sulfate, and either bFGF (A) or EGF (B) to the final concentrations described in the figure. Protamine sulfate and the growth factors were added every other day. The cultures were trypsinized on day 4 and cells counted in a Coulter counter.

DF medium supplemented with both transferrin and insulin were exposed to suboptimal concentrations of either aFGF, bFGF, or optimal concentrations of EGF (Neufeld et al., 1986), together with increasing concentrations of protamine sulfate ranging from 1 to 5 μg/ml. As shown in Figure 1, addition of protamine sulfate alone did not affect cell survival since cultures maintained in DF medium supplemented with insulin and transferrin have similar final cell density regardless of whether they were exposed to protamine or not. Protamine sulfate was growth-inhibitory for cells exposed to either aFGF or bFGF, and its effect was more pronounced on aFGF (half-maximal inhibitory effect at 0.5 μg/ml) than on bFGF (half-maximal inhibitory effect at 2 μg/ml). In contrast, protamine sulfate potentiated the mitogenic effect of EGF. This resulted in a 2-fold stimulation of cell proliferation at a protamine concentration of 5 μg/ml. The effect of protamine sulfate was not due to the sulfate moities, since protamine chloride was just as effective (data not shown).

The effect of a constant protamine sulfate concentration (5 μg/ml) on the proliferation of BHK-21 cells maintained in DF medium supplemented with insulin and transferrin and exposed to increasing bFGF or EGF concentrations is shown in Figure 2. In the absence of protamine sulfate, bFGF was growth-stimulatory at concentrations ranging from 50 pg to 100 ng/ml, with saturation at 10 ng/ml. The concentration required to observe a half-maximal stimulatory effect (ED_{50}) was 400 pg/ml. In the presence of protamine sulfate, bFGF stimulates cell proliferation over the same range of concentrations. However, its potency was strongly decreased since it did not saturate even at 100 ng/ml. The ED_{50} was 20 ng/ml, indicating a 50-fold decrease in biological activity (Fig. 2A).

Opposite results were observed with EGF. In the absence of protamine sulfate, EGF was growth-stimulatory at concentrations ranging from 50 pg/ml to 100 ng/ml, with saturation at 5 ng/ml and an ED_{50} of 300 pg/ml (Fig. 2B). In the presence of protamine sulfate even at concentrations as low as 10 pg/ml, EGF was growth-stimulatory and at saturating concentrations (2 ng/ml), the final cell density of the culture was 2.5-fold higher than in cultures exposed to EGF in the absence of protamine. Potentiation of the mitogenic effect of EGF by protamine sulfate was also reflected in the ED_{50} which was observed at 60 pg/ml, indicating an apparent 5-fold increase in biological activity (Fig. 2B).

The inhibitory effect of protamine sulfate is reversible

In order to test whether BHK-21 cells previously exposed to protamine can be recruited to proliferate actively once exposed to bFGF alone, BHK-21 cells were seeded on gelatin-coated dishes in the presence of DF medium supplemented with transferrin, insulin, and bFGF. Protamine sulfate was either added following the plating of the cells or not. The protamine sulfate and bFGF were added every other day. As shown in Fig. 3, cultures exposed to transferrin, insulin, and bFGF proliferate with an average doubling time of 22 hr, while those exposed to protamine in addition to transferrin, insulin, and bFGF had an average doubling time of 48 hr. When media was renewed on day 2 without protamine being added, cells started proliferating with a growth rate similar to that of cultures never exposed to protamine. Similar results were obtained when media

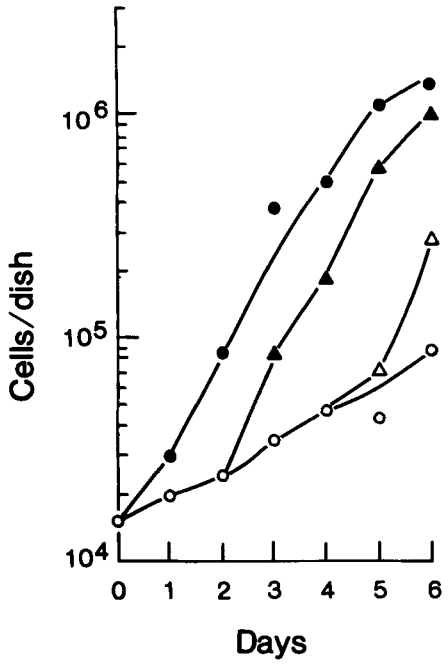

Fig. 3. The inhibition caused by protamine sulfate is reversible. BHK-21 cells (2×10^4) were seeded in 35-mm gelatinized dishes supplemented with insulin and transferrin. Following the plating of the cells, bFGF (10 ng/ml) was added to all of them. One group of cultures (group A) received only bFGF (●), and the other group (group B) received, in addition, 15 μg/ml protamine sulfate (○). Protamine sulfate and bFGF were added every other day. Three dishes of each group were typsinized and counted every day. On day 2, the media in some of the group B cultures was changed to DF medium supplemented with transferrin and insulin containing bFGF without the addition of protamine sulfate (▲). A similar media change was done to other group B cultures on day 4 (△).

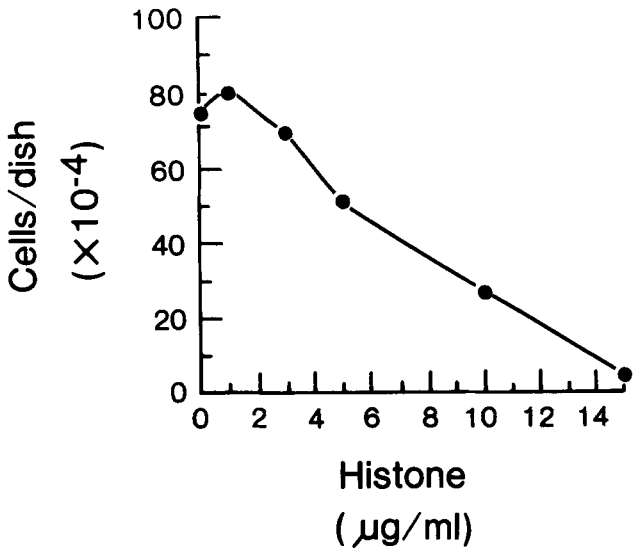

Fig. 4. Histone IIA inhibits the mitogenic activity of bFGF. BHK-21 cells (4×10^4) were seeded in 35-mm gelatinized dishes containing 2 ml of DF medium supplemented with transferrin and insulin. After cells had plated, bFGF (5 ng/ml) and histone IIA were added to triplicate dishes. The cultures were trypsinized and cells counted in a Coulter counter on day 4. Histone and bFGF were added every other day.

was changed on day 4, with the difference that cells had a 1-day lag time before resuming proliferation, with an average doubling time similar to that of cultures never exposed to protamine. This indicates that even when cells were exposed to protamine for 4 days, its effect is completely reversible.

Histone IIA inhibits the mitogenic activity of bFGF on BHK-21 cells

Histones are basic proteins of low molecular weight that bind to DNA and are similar to protamine in this respect. We therefore tested the ability of histone IIA to inhibit the mitogenic effect of bFGF on BHK-21 cells seeded on gelatin-coated dishes and exposed to DF medium supplemented with transferrin, insulin, bFGF, and increasing concentrations of histone ranging from 2 to 15 μg/ml (Fig. 4). The histone concentration that caused a half-maximal inhibition was 7 μg/ml and is thus in the same range as the protamine sulfate concentration that is half-maximal inhibitory under similar conditions (Fig. 1). In contrast, there was no inhibition by cytochrome C, another small basic protein, indicating that for the inhibitory effect, charge alone was not sufficient (not shown).

Effect of protamine on the ability of the ECM to support BHK-21 cell proliferation

The ECM produced by endothelial cells of bovine cornea has been shown in previous studies to mimic the effect of FGF (Gospodarowicz, 1985). In order to analyze whether protamine would have an effect on the ECM similar to that observed with FGF, BHK cells were seeded on ECM-coated dishes and exposed to DF medium supplemented with transferrin, insulin, and increasing concentrations of protamine ranging from 1–20 μg/ml. As shown in Fig. 5A, a marked inhibition of the ability of the ECM to support cell proliferation could be observed at protamine concentrations ranging from 2–20 μg/ml, with near optimal effect at 10 μg/ml. Half-maximal inhibitory effect was observed at a protamine concentration of 2.5 μg/ml. This concentration is very close to that required for decreasing by half the biological activity of bFGF (see Fig. 1). In order to see if pretreatment of the ECM by protamine sulfate would affect its biological activity, ECM-coated dishes were exposed to increasing protamine concentrations ranging from to 40 μg/ml for 2 hr at 4°C. Dishes were then washed with PBS, and BHK-21 cells were seeded in the presence of DF medium supplemented with transferrin and insulin. Since the effect of protamine has been shown to be reversible, and since protamine bound to ECM could dissociate from it, the ability of ECM pretreated with protamine to support cell proliferation was only tested for the first 24 hr using ^{3}H-thymidine incorporation as an assay. As shown in Fig. 5B, protamine-treated ECM had a greatly reduced ability to support initiation of DNA synthesis, as compared to a control not exposed to

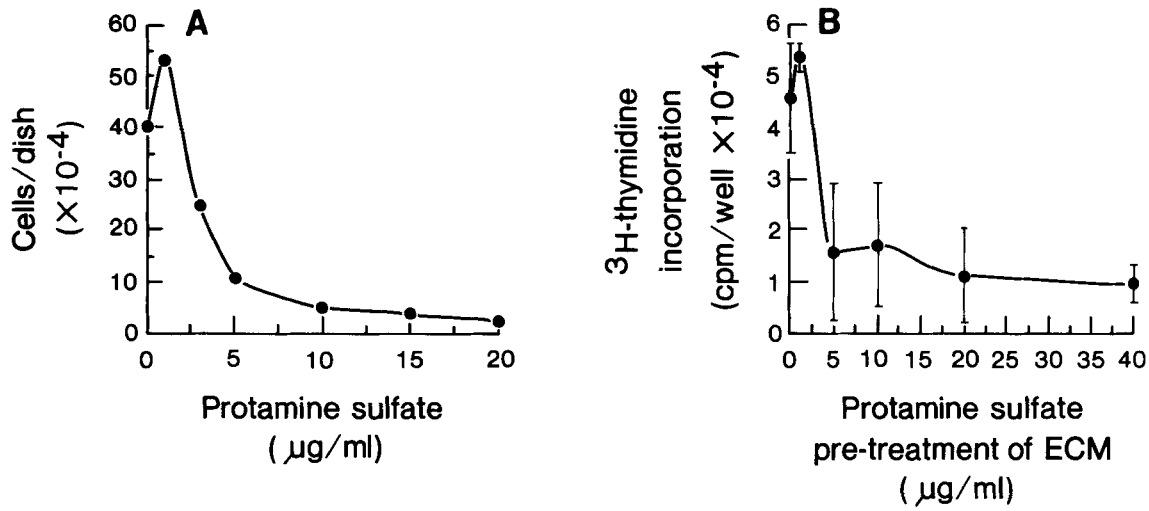

Fig. 5. Protamine sulfate inhibits the proliferation of BHK-21 cells induced by the extracellular matrix produced by bovine corneal endothelial cells. A) BHK-21 cells (4×10^4) were seeded in 35-mm ECM-coated dishes containing 2 ml of DF medium supplemented with transferrin and insulin. The cells were allowed to plate before the addition of protamine sulfate at the indicated concentration. Protamine sulfate was added every other day. The cultures were trypsinized after 4 days and cells counted in a Coulter counter. Points represent the mean of triplicate determinations. B) 35-mm ECM-coated dishes were incubated with the indicated concentrations of protamine sulfate dissolved in PBS for 2 hr at 4°C. At the end of the incubation, the ECM was washed twice with 2 ml of PBS and BHK-21 cells (5×10^4 cells/35-mm dish) were seeded in DF medium supplemented with transferrin and insulin. After 3 hr, ^{3}H-thymidine (1 μCi/ml, final concentration 18 ng/ml) was added. After 24 hr, the cells were washed three times with 2 ml of PBS and lysed with 1 ml of 0.1 M NaOH. Half of that volume was counted in a scintillation counter. The points represent the mean of triplicate determinations, and the bars represent the standard deviation from the mean.

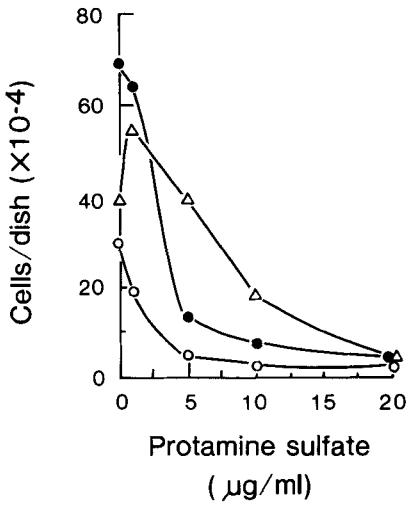

Fig. 6. Protamine sulfate inhibits the bFGF- and ECM-induced proliferation of ABAE cells. ABAE cells (4×10^4) were seeded on gelatin-coated (△) or ECM-coated (●,○) 35-mm dishes containing 2 ml of DMEM supplemented with transferrin and HDL. Following the plating of the cells, bFGF (1 ng/ml) was added (△,●) or not (○) to the cell cultures. At the same time, various concentrations of protamine sulfate were added. BFGF and protamine sulfate were added every other day. After 6 days, the cells were trypsinized and counted with a Coulter counter.

protamine. Near optimal decrease in ^{3}H-thymidine incorporation was observed with ECM pretreated with protamine concentrations as low as 5 μg/ml.

The inhibition of the mitogenic activity of the ECM was not associated with a decrease of cell attachment. This is in agreement with earlier observations that show that the mitogenesis-mediating component of the matrix is not related to cell-attachment-mediating components (Greenburg and Gospodarowicz, 1982).

Effect of protamine on the ability of bFGF and ECM to support the proliferation of vascular endothelial cells

Protamine has been shown to be anti-angiogenic in vivo (Taylor and Folkman, 1982; Majewski et al., 1984). This could reflect its ability to block endothelial cell proliferation supported by an angiogenic factor such as bFGF. In order to test that hypothesis, ABAE cells were seeded on gelatin-coated dishes and exposed to DMEM supplemented with HDL, transferrin, bFGF, and increasing concentrations of protamine ranging from 1–20 μg/ml. As seen in Figure 6, protamine sulfate at a concentration of 20 μg/ml totally blocked cell proliferation supported by bFGF. When cells were seeded on ECM and exposed to DMEM supplemented with HDL and transferrin, protamine was as efficient in blocking cell proliferation induced by the ECM as it was when cells were maintained on gelatin and exposed to bFGF. At 5 μg protamine/ml, total inhibition was observed. In the case of cultures maintained on ECM-coated dishes and exposed to HDL, transferrin, and bFGF, protamine con-

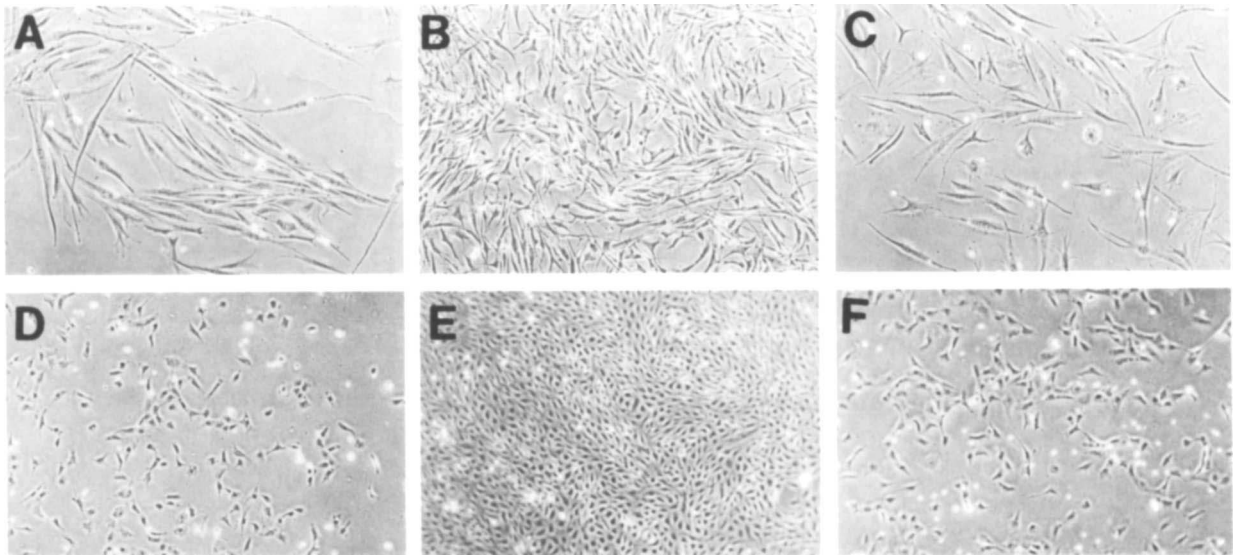

Fig. 7. Protamine sulfate reverses morphological changes induced by bFGF. BHK-21 cells (4×10^4) were seeded in gelatinized 35-mm dishes containing 2 ml of DF medium supplemented with transferrin and insulin (panels A–C). ABAE cells (4×10^4) were seeded in gelatinized 35-mm dishes containing 2 ml of DF medium supplemented with transferrin and HDL (panels D–F). Protamine sulfate and bFGF were added after the cells plated. The cultures in panels A and D did not receive any protamine or bFGF additions. Cultures B and C received 5 ng/ml bFGF, while cultures E and F received 1 ng/ml bFGF. Cultures C and F received, in addition, 15 μg/ml protamine sulfate. BFGF and protamine sulfate were added every other day. The cultures were photographed after 3 days and 6 days in culture, respectively, for BHK-21 and ABAE cells. ×100.

centrations as low as 5 μg/ml almost totally inhibited cell proliferation, and total inhibition of cell proliferation was observed at 20 μg/ml.

Protamine sulfate reverses the morphological changes induced by bFGF

BHK-21 cells exposed to transferrin and insulin alone (Fig. 7A) have the appearance of large and flattened cells with few if any mitotic figures. Addition of bFGF caused them to adopt a rounded morphology, and cells divided rapidly, leading to the formation of confluent cultures composed of small, rounded, and spindly cells (Fig. 7B). Addition of both bFGF and protamine prevented cell proliferation, and the morphological appearance of the cells (Fig. 7C) was similar to that of cultures exposed to transferrin and insulin alone. Similar morphological changes were seen in the case of ABAE cultures maintained under serum-free conditions. While in the presence of transferrin and HDL alone (Fig. 7D), cells were large and flattened; the addition of bFGF alone caused cells to rapidly proliferate, leading to the formation of a cell monolayer composed of small and tightly apposed cells (Fig. 7E). The addition of bFGF and protamine (Fig. 7F) caused cells to assume a morphological appearance similar to that of cultures exposed to transferrin and HDL alone. At high protamine sulfate concentrations (> 20 μg/ml), cytotoxic effects became evident, and the cells became highly vacuolated (not shown).

Protamine sulfate inhibits the binding of ^{125}I-bFGF to BHK-21 cells

Protamine's ability to block the effect of bFGF could either be due to its direct interaction with the mitogen, the resulting protamine-FGF complex being inactive, or due to its ability to block binding of bFGF to cell surface receptors. It is not likely that bFGF forms a stable complex with protamine since bFGF (1 μg/ml) preincubated with protamine (100 μg/ml) was as potent as bFGF alone when tested for its ability to support BHK-21 cell proliferation over a wide range of concentrations ranging from 100 pg to 10 ng/ml. This indicates that the inhibition of the mitogenic activity of bFGF by protamine does not involve a stable interaction between protamine sulfate and the growth factor.

The possibility that protamine interferes with the binding of bFGF with cell surface receptors was investigated using BHK-21 cells, since those cells have been shown to possess a high density of FGF cell surface receptor sites (Neufeld and Gospodarowicz, 1985). Protamine sulfate causes a dose-dependent inhibition of the binding of ^{125}I-bFGF to BHK cells (Fig. 8A). The protamine sulfate concentration that caused a 50% inhibition of binding was 3 μg/ml, which is in good agreement with the concentration that caused a 50% inhibition of the mitogenic effect of bFGF (Fig. 1).

Cross-linking of ^{125}I-bFGF to its BHK cell surface receptor was used in order to find out which of the two receptor species present on BHK cells is interacting with protamine sulfate (Neufeld and Gospodarowicz, 1985, 1986). The experiment shown in Figure 8B clearly demonstrates that protamine sulfate inhibits the cross-linking of ^{125}I-bFGF to both BHK cell surface receptors. The concentrations of protamine sulfate needed for inhibition of the cross-linking of bFGF to the receptor also correlate with the concentrations needed for the inhibition of the biological activity of bFGF. Thus, 1 μg/ml protamine sulfate caused only a partial inhibition of the

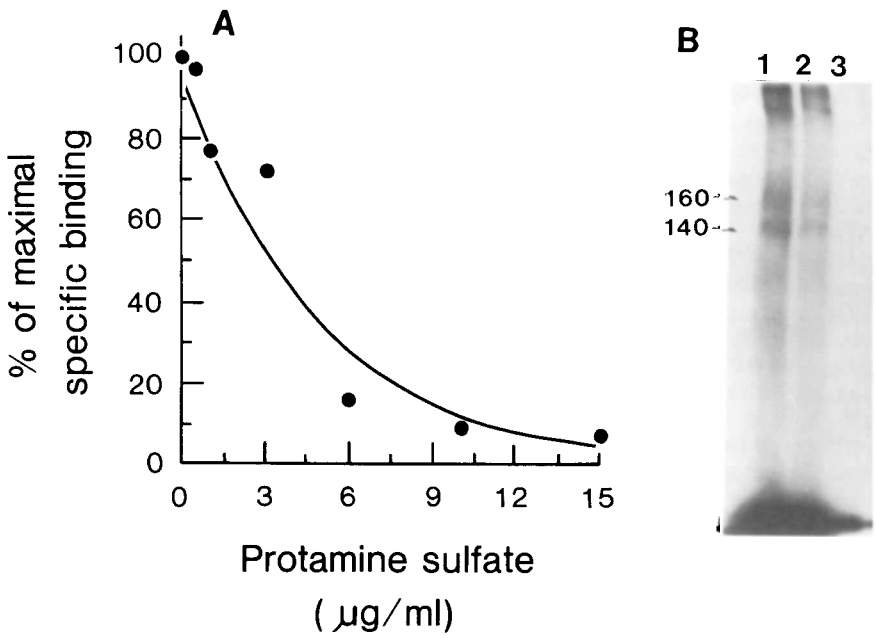

Fig. 8. Protamine sulfate inhibits the binding of ^{125}I-bFGF to its cell surface receptor. A) BHK-21 cells (2×10^4 were seeded per well (Falcon, Micro-test III, 96-well plates) containing serum supplemented medium. The cells were grown to confluence ($6–7 \times 10^4$ cells/well). Binding of ^{125}I-bFGF to the cells in the presence of increasing protamine sulfate concentrations was performed for 2 hr at 4°C. 100% corresponds to 73,000 CPM/well. When the binding was done in the presence of 2 μg/ml of unlabeled bFGF, the non-specific binding that was not displaceable was 18,000 cpm/well. Individual points represent the mean of triplicate determinations. B) Cross-linking of ^{125}I-bFGF (7 ng/ml) to BHK-21 cells. Protamine sulfate was added at the following concentrations during the binding (μg/ml): lane 1, 0; lane 2, 1; lane 3, 10. An aliquot from an equivalent number of cells was loaded on each lane. Samples were reduced and the subsequent SDS/PAGE, fixation, autoradiography, and drying of the gels were done as described previously (Neufeld and Gospodarowicz, 1985). The molecular size markers used were: myosin (M_r = 200,000), B-galactosidase (M_r = 116,000), phosphorylase B (M_r = 92,000), BSA (M_r = 66,000), ovalbumin (M_r = 45,000), and carbonic anhydrase (M_r = 31,000). Autoradiographs are shown of a fixed and dried 6% gel that was exposed 2 days at −70°C.

cross-linking of bFGF to the receptor (Fig. 8B, lane 2), while 10 μg/ml protamine sulfate caused almost complete inhibition (Fig. 8B, lane 3).

DISCUSSION

Angiogenesis in vivo is the result of a complex and coordinated cascade of events which involves increased activity/induction of various cellular enzymes involved in basement membrane degradation, increased cell migratory activity, and induction of cell proliferation. Protamine has been shown in previous studies to be anti-angiogenic (Taylor and Folkman, 1982; Majewski et al., 1984). Although the molecular basis for this activity is presently unknown, it is conceivable that protamine could inhibit new blood vessel formation by preventing the occurrence of one or multiple events during angiogenesis. Particularly attractive is the possibility that protamine could inhibit the mitogenic activity of angiogenic factors.

The purification and structural characterization of angiogenic factors such as aFGF or bFGF, as well as our understanding of their interaction with specific cell surface receptors, provide us with a model for the study in vitro of the anti-angiogenic activity of protamine, as well as an understanding of the molecular mechanism underlying it. Using both BHK-21 cells and vascular endothelial cells, our results demonstrate that protamine can act as an inhibitor of both bFGF and aFGF and prevent the morphological changes induced by these mitogens. Since both aFGF and bFGF have been shown in previous studies to be angiogenic in vivo, it is likely that within the in vivo context, protamine could act in similar ways on angiogenic factors related or identical to FGF.

Previous studies have also shown that the basement membrane produced by cultured cells could support the proliferation and differentiation of vascular endothelial cells (Gospodarowicz and Tauber, 1980). Basement membrane materials are also angiogenic in vivo (West et al., 1985). It is, therefore, of interest to observe that protamine can also inhibit the mitogenic activity of ECM produced by corneal endothelial cells. This indicates that angiogenic factors, either FGF itself or agents closely related to it, could be associated with the ECM. Protamine bound to the ECM might block the interaction of ECM-associated FGF with receptors located on the basal cell surface.

The ability of protamine to inhibit cell proliferation induced by bFGF could result from a direct action on the cell itself, making it refractory to growth factors in general. That, however, is unlikely since protamine, while inhibiting the mitogenic effect of aFGF and bFGF, is potentiating that of EGF. Therefore, protamine can affect the biological activity of various growth factors in

opposite ways. Equally unlikely is the possibility that the inhibitory effect of protamine could result from the formation of a stable complex with bFGF since preincubation of this growth factor with protamine at 100-fold molar excess did not inhibit its mitogenic activity. More likely, as our results indicate, protamine inhibits the binding of bFGF to cell surface receptors, therefore providing a molecular basis for our understanding of protamine action.

In contrast to its effect on FGF, protamine sulfate potentiates the mitogenic activity of EGF even in presence of saturating EGF concentrations. This indicates that protamine might interact with cellular sites not associated with the receptors for FGF or EGF. The mechanism by which protamine sulfate causes this effect has not been further studied. It is interesting to note that protamine sulfate has been shown to inhibit the binding of platelet derived growth factor to its receptor (Huang et al., 1982), suggesting that the platelet derived growth factor and FGF receptors might share some similarity, not shared by the EGF receptor.

In conclusion, it should be noted that the anti-angiogenic activity of protamine, coupled with its FGF-inhibiting activity, suggests that FGF-inhibitory substances might also be effective angiogenesis inhibitors. The identification and characterization of the receptor binding domains of bFGF and aFGF, as well as that of protamine sulfate, might therefore aid the development of a new anti-cancer family of drugs.

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