Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation

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Non-enzymic glycosylation of basic fibroblast growth factor (bFGF, FGF-2) has recently been demonstrated to decrease the mitogenic activity of intracellular bFGF. Loss of this bioactivity has been implicated in impaired wound healing and micro-angiopathies of diabetes mellitus. In addition to intracellular localization, bFGF is also widely distributed in the extracellular matrix, primarily bound to heparan sulphate proteoglycans (HSPGs). Nonetheless, it is not clear if non-enzymic glycosylation similarly inactivates matrix-bound bFGF. To investigate this, we measured the effect of non-enzymic glycosylation on bFGF bound to heparin, heparan sulphate and related compounds. Incubation of bFGF with the glycosylating agents glyceraldehyde 3-phosphate (G3P; 25 mM) or fructose (250 mM) resulted in loss of 90 % and 40 % of the mitogenic activity of bFGF respectively. Treatment with G3P and fructose also decreased the binding of bFGF to a heparin column. If heparin was added to bFGF prior

to non-enzymic glycosylation, the mitogenic activity and heparin affinity of bFGF were nearly completely preserved. A similar protective effect was demonstrated by heparan sulphate, low-molecular-mass heparin and the polysaccharide dextran sulphate, but not by chondroitin sulphate. Whereas non-enzymic glycosylation of bFGF with G3P impaired its ability to stimulate *c-myc* mRNA expression in fibroblasts, no such impairment was noticeable when bFGF was glycosylated in the presence of heparin. Taken together, these results suggest that HSPG-bound bFGF is resistant to non-enzymic glycosylation-induced loss of activity. Therefore, alteration of this pool probably does not contribute to impaired wound healing seen in diabetes mellitus.

Key words: diabetes mellitus, endothelial cell, extracellular matrix, glyceraldehyde 3-phosphate, glycosaminoglycan.

INTRODUCTION

Impaired wound healing and micro- and macro-vascular angiopathies are common complications of diabetes mellitus [1,2]. One mechanism that may contribute to these sequelae is the alteration of angiogenic growth factors by the process of non-enzymic glycosylation. Chronic hyperglycaemia has long been known to lead to chemical modification of proteins such as albumin, collagen and haemoglobin [3,4]. Despite this, only recently has a potential role for non-enzymic glycosylation in the modulation of growth factor activity been proposed. A recent report by Giardino et al. [5] has demonstrated clearly that non-enzymic glycosylation reduces the activity of basic fibroblast growth factor (bFGF, FGF-2). This study primarily focused on the capacity of non-enzymic glycosylation to influence intracellular bFGF. However, significant quantities of bFGF are also bound to the glycosaminoglycan (GAG), heparan sulphate, in the extracellular matrix (ECM) [6–8]. The capacity of non-enzymic glycosylation to alter this GAG-bound pool is yet to be determined.

There are compelling reasons to investigate the effect of non-enzymic glycosylation on GAG-bound bFGF. This pool of bFGF has multiple potential functions, including the regulation of cellular differentiation and proliferation and the early initiation of cellular repair after injury [9,10]. The ECM may also act as a reservoir capable of providing rapid release of bFGF after injury [11–13]. Given its biological potential, mitigation of

the activity of the GAG-bound pool of bFGF by non-enzymic glycosylation might contribute to sequelae of diabetes such as impaired wound healing.

The interaction between bFGF and the ECM is primarily mediated by matrix heparan sulphate proteoglycans (HSPGs). bFGF contains discrete heparin-binding regions which bind with high affinity to HSPG and heparin-like GAGs [14]. Glycosaminoglycans similar to those in the ECM and on the cell surface, including heparin and heparan sulphate, have been shown to protect bFGF from inactivation due to heat, acid and proteolytic degradation [15–17]. To date, no information is available on the capacity of ECM-related GAGs to protect bFGF from non-enzymic glycosylation. Therefore, we investigated the potential for heparin and heparan sulphate to protect bFGF from non-enzymic glycosylation-induced inactivation.

EXPERIMENTAL PROCEDURES

Materials

bFGF was obtained from R&D Systems (Minneapolis, MN, U.S.A.). All GAGs, glycosylating agents, cell-culture media and heparin-agarose columns were procured from Sigma Chemicals (St. Louis, MO, U.S.A.). Na¹²⁵I was obtained from Dupont (Boston, MA, U.S.A.). For cell-proliferation assays, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt].

Abbreviations used: bFGF, basic fibroblast growth factor; GAG, glycosaminoglycan; ECM, extracellular matrix; HSPG, heparan sulphate proteoglycan; PMS, phenazine methosulphate; G3P, glyceraldehyde 3-phosphate; HUVEC, human umbilical vein endothelial cell; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; MTS, T3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt.

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(4-sulphophenyl)-2*H*-tetrazolium, inner salt] powder (Owen's reagent) was obtained from Promega (Madison, WI, U.S.A.) and phenazine methosulphate (PMS) powder from Sigma. Dextran sulphate (500 kDa) was a product of Fischer Scientific (Fair Lawn, NJ, U.S.A.).

Glycosylation

bFGF was glycosylated essentially as described by Giardino et al. [5]. bFGF (1 µg/ml) was incubated in 200 mM phosphate buffer, pH 8.0, at 37 °C with either a glycosylating agent alone or a glycosylating agent in combination with a GAG or dextran sulphate. Glycosylating agents included fructose (250 mM) and glyceraldehyde 3-phosphate (G3P; 25 mM). Incubations were performed for 3 (G3P) or 24 h (fructose). The GAGs tested included heparin sulphate, heparan sulphate, chondroitin sulphate (ratio of A/B, 70:30) and low-molecular-mass heparin.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA, U.S.A.) and cultured under standard conditions ideal for propagating human endothelial cells. HUVECs were maintained in gelatinized flasks in Dulbecco's minimum essential medium (DMEM)/Ham's F12 (1:1) containing 15% fetal bovine serum (FBS), heparin (50 µg/ml) and bovine hypothalamic extract (150 µg/ml) [18]. BHK-21 fibroblasts were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and maintained in DMEM/Ham's F12 (1:1) medium containing 10% FBS.

Endothelial cell proliferation assay

Between the 3rd and 5th passages, HUVECs were plated on to gelatin-coated 96-well plates at a cell density of 2000 cells/well. HUVEC monolayers were incubated at 37 °C for 72 h with control or treated bFGF in DMEM/Ham's F12 (1:1) medium containing 10% FBS and heparin (50 µg/ml). Cell proliferation was quantified as described previously using a colorimetric assay in which a formazan product is produced in proportion to cell number [13,19]. Briefly, a stock solution of PMS (1 mg/ml in PBS) was diluted 1/20 in MTS (2 mg/ml in DMEM, pH 6.7). The MTS/PMS mixture was further diluted 1/5 (v/v) in basal medium and added to 96-well culture plates. After incubating for 4 h at 37 °C, absorbance was measured at 492 nm and a percentage maximal proliferation calculated as an absorbance ratio of sample wells to wells containing control (non-glycosylated) bFGF. Routinely, maximal proliferation of HUVECs was observed at the concentration of 20 ng/ml bFGF.

In proliferation experiments designed to test the protective effect of heparin and other GAGs on glycosylation-induced loss of bioactivity of bFGF, the GAGs were not separated from the post-glycosylation mixture because the concentration of heparin included in the growth medium was several orders of magnitude greater than that of the GAGs. Therefore the small concentrations of GAGs would have little effect on the observed effects in our proliferation assays.

Iodination of bFGF

Recombinant bFGF was iodinated using chloramine-T as described by Benezra et al. [11]. After iodination, ¹²⁵I-bFGF was purified using a heparin-agarose column equilibrated with 10 mM Tris, pH 7.2. ¹²⁵I-bFGF was eluted from the column with 2 M NaCl containing 0.1% BSA and was then dialysed against PBS. Specific radioactivity of ¹²⁵I-bFGF after iodination was

typically 10⁷ c.p.m./µg. Iodinated bFGF was stored at 4 °C and used within 1 week.

Heparin affinity experiments

¹²⁵I-bFGF (5 × 10⁵ c.p.m.) was incubated with either fructose alone or G3P alone, or each in the presence of heparin as described above. Treated samples were loaded on to a heparin-agarose column equilibrated with 10 mM Tris/0.4 M NaCl buffer, pH 7.2. After washing the column with Tris/NaCl buffer, ¹²⁵I-bFGF was eluted in 1 ml fractions with a linear NaCl gradient (0.4–2.0 M) with a flow rate of 0.5 ml/min using a fraction collector (Bio-Rad, Hercules, CA, U.S.A.). ¹²⁵I-bFGF was eluted at 2 M NaCl.

Northern-blot analysis

BHK-21 cells were grown to semi-confluence in 15 cm diameter plates with DMEM/Ham's F12 medium containing 10% FBS at 37 °C in 5% CO₂. On the day of the experiment, the monolayers were washed once with serum-free medium and incubated with test reagents for 60 min at 37 °C. At the end of the incubation period, cells were washed once with PBS and total RNA was extracted with guanidinium isothiocyanate (4.2 M guanidinium isothiocyanate/18 mM Sarkosyl/0.7% β-mercaptoethanol) and separated by isopycnic centrifugation over CsCl. Total RNA was then electrophoresed on a formaldehyde denaturing agarose gel and transferred to a nylon membrane (Schleicher and Schuell, Keene, NH, U.S.A.) by capillary transfer. The membranes were then hybridized with [³²P]dCTP-labelled cDNA probes for *c-myc* and β-actin.

RESULTS

Mitogenic activity of glycosylated bFGF

bFGF incubated with glycosylating sugars showed diminished HUVEC proliferative activity compared with bFGF incubated in buffer alone. Treatment with G3P (25 mM) resulted in a 90% loss of proliferative activity. Fructose (250 mM) treatment of bFGF resulted in an approximately 40% loss of proliferative activity (Figure 1). The differential capacity of these two sugars

Figure 1 Effect of non-enzymic glycosylation on the proliferative activity of bFGF

Recombinant bFGF was incubated in a 37 °C water bath with DMEM (24 h) or the glycosylating agents fructose (24 h) and G3P (3 h). After non-enzymic glycosylation, increasing concentrations of bFGF were added to HUVECs. Cell proliferation was determined after 3 days. Results are depicted as a percentage of maximal HUVEC proliferation.

Figure 2 Protective effect of heparin sulphate against non-enzymic glycosylation

bFGF was incubated for 3 h at 37 °C with DMEM, G3P alone, or G3P combined with heparin. After treatment, increasing concentrations of bFGF were added to HUVEC cultures. Proliferative activity of bFGF was determined after 3 days.

to alter bFGF function probably reflects the glycosylating potential of each sugar, which in turn reflects the stability of the aldose isoform. Our results were consistent with those reported by Giardino et al. [5]. To account for the loss of activity due to heat alone, buffer-treated bFGF was compared with bFGF that had not undergone incubation. A 24 h incubation at 37 °C typically resulted in loss of 20–30% of proliferative activity, whereas a 3 h incubation produced no discernable loss of activity. In order to achieve maximal glycosylation without loss of activity due to heat inactivation, G3P was utilized as the primary glycosylating agent for the remaining studies, as G3P requires only a 3 h incubation period.

Protective effect of heparin

Although non-enzymic glycosylation decreases the mitogenic activity of bFGF, it is unclear whether the presence of GAGs such as heparin impedes this process. When bFGF was combined with heparin prior to the addition of G3P, the proliferative activity of bFGF was comparable with non-glycosylated bFGF (Figure 2). The protective effect of heparin was concentration dependent and was maximal at or above 50 µg/ml. Addition of heparin subsequent to non-enzymic glycosylation did not restore the mitogenic activity of bFGF. Heparin also fully protected the mitogenic activity of bFGF treated with fructose (results not shown).

Protective effect of heparan sulphate and other GAGs

To determine if the protective effect of heparin was shared by other sulphated GAGs, bFGF was glycosylated with G3P in the presence of several other GAGs. Heparan sulphate and low-molecular-mass heparin showed protective activity similar to heparin sulphate (Figure 3). Similarly, the highly sulphated polysaccharide dextran sulphate fully preserved the activity of bFGF at all concentrations tested. In contrast, the GAG chondroitin sulphate displayed minimal capacity to protect bFGF from non-enzymic glycosylation (Figure 4). A potential explanation for the protective effect of the GAGs in our cell-culture system is that the higher concentration of heparin included in our culture system would displace the GAGs and that the

Figure 3 Protective effect of heparin-like GAGs

bFGF was combined with DMEM or varying concentrations of heparin sulphate, heparan sulphate or low-molecular-mass (LMW) heparin and then incubated with the glycosylating agent G3P (3 h, 37 °C). After non-enzymic glycosylation, the proliferative activity of a constant concentration of treated bFGF (10 ng/ml) was determined using a HUVEC proliferation assay. Results are depicted as the percentage of proliferative activity of native bFGF (10 ng/ml).

effect seen is primarily due to the protective effect of heparin. Such a scenario is highly unlikely because chondroitin sulphate failed to protect bFGF from glycosylation-induced damage. Furthermore, heparin in the culture medium did not reverse the loss of activity when bFGF was glycosylated by G3P alone. The strength of a GAG to protect the growth factor from loss of activity when glycosylated is perhaps reliant on its strength of binding to specific sites on bFGF that are amenable to glycosylation.

Heparin affinity of bFGF

One potential mechanism by which non-enzymic glycosylation may diminish the activity of bFGF is by altering the affinity of

Figure 4 Protective effect of chondroitin sulphate and dextran sulphate

bFGF was combined with DMEM or varying concentrations of heparin sulphate, chondroitin sulphate or dextran sulphate and then incubated with the glycosylating agent G3P (3 h, 37 °C). After non-enzymic glycosylation, the proliferative activity of a constant concentration of treated bFGF (10 ng/ml) was determined using a HUVEC proliferation assay. Results are shown as the percentage of maximal proliferative activity of bFGF (10 ng/ml).

Figure 5 Heparin-binding activity of G3P-treated bFGF

^{125}I -bFGF was combined with DMEM or heparin sulphate and then incubated with the glycosylating agent G3P (3 h, 37 °C). After non-enzymic glycosylation, ^{125}I -bFGF was applied to a heparin-agarose column and eluted with a NaCl gradient (dashed line, 0.4–2.0 M).

Figure 6 Protective effect of heparin on the loss of *c-myc* mRNA expression induced by non-enzymic glycosylation of bFGF

BHK-21 cells were treated for 30 min at 37 °C with one of the following: (i) media control; (ii) bFGF glycosylated with G3P in the presence of heparin; (iii) bFGF glycosylated using G3P; (iv) bFGF plus heparin; and (v) bFGF alone. Steady-state levels of *c-myc* mRNA expression were determined by Northern-blot analysis as described in the Experimental procedures section.

bFGF for heparin, thereby interfering with the synergy between bFGF and heparin. Conversely, the protective effect of heparin during non-enzymic glycosylation may be due to preservation of heparin-binding sites of bFGF. To address this possibility

we compared the heparin-binding capacity of glycosylated ^{125}I -bFGF with that of ^{125}I -bFGF protected during glycosylation by soluble heparin. Glycosylated, heparin-protected and control ^{125}I -bFGF each were eluted from a heparin-agarose column at 2.0 M NaCl. Glycosylation with G3P resulted in 94 % decrease in the amount of ^{125}I -bFGF bound to the column, compared with samples incubated in buffer alone (Figure 5). The addition of heparin during glycosylation almost completely preserved total column binding of G3P-treated ^{125}I -bFGF. Soluble heparin therefore directly preserves the heparin-binding activity in addition to the mitogenic activity of bFGF. The inability of glycosylated bFGF to bind to the heparin-agarose column is also reflected in the higher radioactive counts (84 %) in the flow-through fractions (fractions 1–8). In contrast, only 25 % of the total c.p.m. added to the column was found in the flow-through fractions in unglycosylated and heparin-protected bFGF samples.

c-myc activation

One of the FGF-receptor-mediated effects of bFGF is the activation of *c-myc* transcription. To determine if non-enzymic glycosylation alters the capacity of bFGF to interact with the FGF receptor, we measured the induction of *c-myc* transcripts by glycosylated and heparin-protected bFGF. BHK-21 fibroblasts were used for these studies because of the relatively high number of surface FGF receptors in this cell type. We found the proliferation of BHK-21 cells in response to G3P-glycosylated and heparin-protected bFGF to be almost identical to that of HUVECs (results not shown). Figure 6 shows the dramatic loss of *c-myc* activation by bFGF glycosylated with G3P. Heparin preserves the capacity of bFGF to stimulate *c-myc* transcripts. These results suggest that glycosylation of bFGF

alters its capacity to activate the FGF receptor, and support the hypothesis that heparin protects bFGF by preserving its capacity to bind to heparin in a configuration that facilitates activation of the FGF receptor.

DISCUSSION

We report the novel finding that heparin and heparan sulphate protect bFGF from loss of activity due to non-enzymic glycosylation. To demonstrate this, we used a model of non-enzymic glycosylation *in vitro* described recently by Giardino et al. [5]. Using this model, we found that a 3 h exposure of recombinant bFGF to G3P results in profound loss of proliferative and heparin-binding activity of bFGF. When added prior to G3P incubation, heparin and heparan sulphate nearly completely preserved the activity of bFGF. Addition of heparin after G3P exposure did not restore bFGF activity. Heparin and heparan sulphate therefore appear to prevent, but not reverse, the critical alterations of bFGF induced by non-enzymic glycosylation.

A protective role for heparin against conditions other than non-enzymic glycosylation has previously been reported. Heparin has been shown to protect bFGF from heat and acid mediated inactivation [15]. Both heparin and endothelial cell-derived heparan sulphate have also been shown to protect bFGF from inactivation due to proteases such as trypsin and plasmin [16,20]. Coltrini et al. compared various GAGs and found that heparin provided the optimal protective effect against proteolytic inactivation [17]. This report also found that highly sulphated polyanions like dextran sulphate duplicate the protective effect of heparin. Our results similarly demonstrate that heparin, heparan sulphate, low-molecular-mass heparin and dextran sulphate preserve the bioactivity of bFGF from non-enzymic glycosylation in a concentration-dependent manner. Chondroitin sulphate, however, offers little protection against non-enzymic glycosylation. Our results provide further support for a specific physiologic role for heparin-like molecules in protecting bFGF.

The mechanism by which heparin protects the bioactivity of bFGF from non-enzymic glycosylation is not known. One possibility is that heparin protects critical heparin-binding domains of bFGF from non-enzymic glycosylation and, in so doing, preserves the potential for bFGF and heparin to interact jointly with the cell-surface FGF receptor. bFGF contains multiple discrete heparin-binding regions that are rich in lysine and are therefore susceptible to non-enzymic glycosylation [21–24]. Alternatively, heparin and other GAGs may also help to maintain the active configuration of bFGF even in the presence of the glycosylating sugars. These potential mechanisms are not mutually exclusive and it is distinctly possible that both mechanisms contribute to the protective effect of heparin and the other GAGs.

bFGF-mediated receptor activation and mitogenic activity have been well described as requiring heparin-like molecules as co-factors [25–27]. Heparin may act by binding bFGF in an orientation that facilitates FGF-receptor dimerization and activation [24,26,28]. According to this model, loss of affinity of bFGF for heparin would limit the capacity of bFGF to stimulate FGF-receptor-mediated cell signalling. In order to test the possibility that non-enzymic glycosylation of bFGF impairs intracellular signalling, we examined steady-state levels of *c-myc* RNA in BHK-21 cells in response to control and G3P-treated bFGF. Treatment of bFGF with G3P resulted in significant reduction in the steady-state levels of *c-myc* mRNA. The loss of cell signalling, mitogenicity and heparin-binding activity of bFGF suggests that non-enzymic glycosylation interferes with the interaction between bFGF and its receptor. The fact that

heparin is able to preserve the bioactivity of bFGF suggests that non-enzymic glycosylation modifies critical residues within the heparin-binding regions of bFGF. This alteration may prevent heparin-mediated bFGF dimerization and FGF-receptor activation.

One of our goals in this study was to determine if bFGF in the ECM is susceptible to non-enzymic glycosylation. Giardino et al. [5] reported recently that non-enzymic glycosylation of intracellular bFGF occurs in cells containing high intracellular glucose levels. We postulated that, since a large pool of bFGF is distributed in the ECM, and since long-lived ECM proteins are often affected in diabetes, non-enzymic glycosylation of ECM-bound bFGF might diminish the activity of this pool and potentially lead to various sequelae of diabetes. bFGF binds to the ECM by binding to specific HSPGs [14]. Binding of bFGF to heparin and HSPGs closely models the interaction between bFGF and ECM HSPGs. Our finding that heparin and heparan sulphate protect bFGF suggests that non-enzymic glycosylation is unlikely to significantly alter the pool of bFGF bound to HSPGs in the ECM.

The potential for the ECM to prevent non-enzymic glycosylation of bFGF suggests an important role for the ECM in conditions such as diabetes. The ECM-bound pool of bFGF has numerous potential functions. For example, ECM-bound bFGF mediates cell proliferation and differentiation [9]. Injured cells are able to utilize ECM-bound bFGF to initiate repair [10]. Enzymes such as thrombin and heparanases release active HSPG–bFGF complexes from the ECM, allowing diffusion of these angiogenic compounds [11,12,29]. These functions have led to speculation that ECM-bound bFGF mediates processes such as wound repair and angiogenesis [14]. If the intracellular pool of bFGF is in fact affected by non-enzymic glycosylation, the ECM-bound pool of bFGF might gain even greater importance in the diabetic patient. However, it is also possible that alteration of intracellular bFGF by non-enzymic glycosylation limits the capacity of bFGF to bind to the extracellular matrix and therefore diminishes the relative size of the extracellular pool of bFGF. In fact, loss of bioactivity of intracellular bFGF by non-enzymic glycosylation has already been reported by Giardino et al. [5]. However, a full assessment of the potential for non-enzymic glycosylation of bFGF in the diabetic condition will require analysis *in vivo*.

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