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Interaction of specifically chemically modified dextrans with transforming growth factor β1: potentiation of its biological activity

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Abstract

Transforming growth factor β (TGFβ), a potent multifunctional cytokine, is well known to demonstrate heparin binding ability. This study investigated the binding capacity of heparin-like family of chemically modified dextrans to TGFβ1. Dextran derivatives with various substitution contents in carboxymethyl, benzylamide and sulfate groups were evaluated using a gel mobility shift assay. This structure–function study indicated that a synergistic role of benzylamide and sulfate substituents resulted in an optimal interaction with the growth factor. The effect of these polymers on the biological response of TGFβ1 was assessed using mink lung epithelial cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct (PAI/Luc). When the growth factor was mixed with 250 μg/mL of carboxymethyl-benzylamide-dextran (DCMB) or carboxymethyl-benzylamide-sulfate-dextran (DCMBSu), the luciferase gene expression was enhanced. Only polymers exhibiting TGFβ1 binding demonstrated a biological potentiating effect. However, this effect was strongly amplified as the cell plating time increased (35-fold increase with a 2 days plating time versus 1.1-fold increase with a 4 hr plating time at a 0.25 ng/mL concentration of TGFβ1). TGFβ1 induced the PAI/Luc construct in a dose-dependent fashion but its effect diminished when added to cells previously cultured for 24 and 48 hr. The results indicated that the potentiating effect required a complex formation between TGFβ1 and polymers, the action of which seeming to locally maintain TGFβ1 in an active form. TGFβ isoforms playing a key role in the process of bone repair, specifically designed functionalized dextrans could potentiate the *in vivo* TGFβ1 biological effect and be used in the field of wound healing applications.

Keywords: Functionalized dextrans; TGFβ1; Interactions; Gel shift assay; Luciferase reporter construct

1. Introduction

TGFβ1 is a member of a larger superfamily of growth factors. These ubiquitous molecules include subfamily members such as activins, inhibins, growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs) and the five identified isoforms of TGFβ. TGFβ1 to TGFβ3 are known to be important in the regulation of mammalian tissues. All TGFβs are dimeric proteins, with homodimeric and heterodimeric forms identified and having the ability to interact with several types of specific signaling cell surface receptors which activate intracellular substrates, including Smad proteins [1,2]. Type I and type II serine/threonine kinase transmembrane receptors are most important for signal transduction, whereas TGFβ type III receptor (also termed betaglycan) regulate the binding of ligands to the types I and II receptors [3–5].

The multifunctional effects of TGFβ1 are cell growth regulation, matrix production stimulation and immune system inhibition [6]. Major sources of TGFβ1 in the body include platelets, bone and serum [7–9]. However, all of these sources contain latent, not active TGFβ. Active TGFβ must be dissociated from a secreted latent complex to exhibit a biological effect through binding to receptors. The skeletal matrix is probably the largest reservoir of

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Abbreviations: DCM, carboxymethyl dextran; DCMB, carboxymethyl-benzylamide-dextran; DCMBSu, carboxymethyl-benzylamide-sulfate-dextran; DCMSu, carboxymethyl-sulfate-dextran; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MLEC, mink lung epithelial cells; PAI-1, plasminogen activator inhibitor-1; PAI/Luc, luciferase expression vector driven by PAI-1 promoter; PBS, phosphate buffer saline; TGFβ, transforming growth factor β.

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TGFβ in the organism [10]. Such an abundant growth factor with such potent effects on cells must be tightly regulated and this regulation is achieved through latency [11,12]. *In vitro* and *in vivo* studies have indicated a major role of TGFβ1 in the bone remodeling process by recruitment and proliferation of osteoblast precursors [13]. Moreover, exogenous TGFβ has been used in several critical size defects models of bone regeneration and fracture healing, with most of the studies showing increased bone formation and mechanical stability [14]. Excess amount of TGFβ has never been shown detrimental to bone healing or bone formation, while it has demonstrated some negative effects, inducing scarring and fibrosis in other tissues.

TGFβ1 has been shown to possess an heparin binding ability *in vitro* [15]. Such interaction with this natural heterogeneous anionic polysaccharide protects TGFβ1 from proteolytic degradation *in vitro* [16], and also prevents the formation of inactive complexes with α_2-macroglobulin (α_2M) [17,18]. Previous investigations have demonstrated that an heparin-like family of dextran derived polymers is also able to protect TGFβ1 from the enzymatic degradation [19]. This family encompasses a wide range of dextran derivatives substituted with carboxymethyl (CM), benzylamide (B) and sulfate groups (Su). Depending on the relative proportions of each chemical group, these soluble polysaccharides display numerous biological properties through their interaction with specific protein domains (for review, see [20]). Some of these polymers exhibit a range of biological properties that make them a synthetic match of heparin. In particular, these active dextran derivatives interact with numerous heparin binding growth factors as fibroblast growth factors (FGFs), platelet derived growth factor (PDGF) and TGFβ1. A dextran substituted with CM and B chemical groups increased the electrophoretic mobility of FGF2, TGFβ1 and PDGF and displaced these growth factors from their receptors [21,22]. Similar to heparin and heparan sulfate, some of these synthetic polysaccharides protected FGFs from chemical and proteolytic degradation *in vitro* [23] and potentiated the mitogenic activity of FGFs on Chinese hamster lung fibroblasts [24].

The present study investigated the ability of well-characterized dextran derivatives to interact with TGFβ1, and thereby modulate its biological activity. In the first part, the effect of various polymeric chemical substitutions was investigated to define the most efficient chemical composition of dextran derivatives that can bind to the growth factor. Then, the effect of these polymers on the biological response of TGFβ1 was assessed using a convenient bioassay based on the ability of TGFβ to induce plasminogen activator inhibitor-1 (PAI-1) expression. TGFβ1 bound to receptors of mink lung epithelial cells (MLEC) transfected with PAI-1 promoter-luciferase construct (PAI/Luc) resulting in a dose-dependent increase of luciferase activity [25].

2. Materials and methods

2.1. Materials

Dextran T40 (Mn = 28 800, Mw = 40 400, Batch 228608) was purchased from Pharmacia. All chemicals and solvents were of analytical grade. Porcine intestine heparin was purchased from Sanofi and was characterized by the molecular weight of 18,000 g/mol. TGFβ1 was purchased from Amersham biotech and antibodies (anti-human TGFβ1 and alkaline phosphatase-labeled anti-rabbit-IgG-conjugated) were purchased from Promega. MLEC was a generous gift of Dr. Rifkin (New York University Medical Center, NY, USA). They have been transfected with a PAI-1 promoter-luciferase construct (PAI/Luc) [25].

2.2. Dextran derivatives preparation

Water-soluble dextran derivatives were obtained by statistical substitution of native dextran polymer with three different chemical groups. Polymers were prepared as previously described [26,27]. Briefly, the synthesis involved three steps: statistical carboxymethylation of hydroxyl groups on glycosyl units (CM), benzylamidation of the carboxylic groups (B), and/or sulfatation on free hydroxyl groups (Su). Four groups of dextran derivatives had been synthesized including (i) dextran substituted with CM groups (DCM), (ii) dextran substituted with CM groups and increasing substitutions of B groups (DCMB), (iii) dextran substituted with CM, B and increasing substitutions of sulfate groups (DCMBSu), and (iv) dextran only substituted with CM and Su groups (DCMSu) (Table 1). Polymers named DCMB1.1 to DCMB1.3 were benzylamidated from the same precursor DCM and polymers named DCMBSu1.1 to DCMBSu1.3 were sulfated from the same precursor DCMB. The polymers were then purified by ultrafiltration and lyophilized. The chemical composition was determined by acidic titration and

Table 1
Degree of substitution (d.s.)[a] of carboxymethyl (CM), benzylamide (B) and sulfate (Su) groups on water soluble derivatized dextrans

	d.s. of CM	d.s. of B	d.s. of Su
DCM	0.98	–	–
DCMB1.1	0.92	0.15	–
DCMB1.2	0.76	0.31	–
DCMB1.3	0.64	0.43	–
DCMB2	0.65	0.29	–
DCMBSu1.1	0.76	0.38	0
DCMBSu1.2	0.75	0.37	0.64
DCMBSu1.3	0.74	0.37	1.08
DCMBSu2	0.75	0.35	0.86
DCMSu	1.04	0	0.70

[a] The d.s. corresponds to the average number of substituent per glycosyl unit.

elemental analysis of nitrogen and sulfur contents. The molecular weight of each polymer determined by HPSEC were ranging from 75,000 to 90,000 g/mol.

2.3. Gel mobility shift assay

The dextran derivatives effect on the electrophoretic mobility of TGFβ1 was analyzed by agarose gel electrophoresis [28]. TGFβ1 (typically 60 nM) was mixed for 2 hr at 4° with various amounts of polymers in 10 μL of glutamate buffer (5 mM glutamate, 0.5% sucrose, 2.5% glycine, pH 4.5). After adding 2 μL of glycerol 75% (v/v)/ Bromophenol Blue 1% (w/v), the mixture was then loaded into 0.8% (w/v) agarose gel prepared in running buffer (125 mM sodium acetate, 50 mM Tris–HCl, pH 6.5). Typically, electrophoresis was performed at 200 mA for 4 hr at 4°.

Blotting and immunodetection were performed according to standard protocols [29]. The blotting buffer contained 25 mM Tris base, 192 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS pH 9.0. Proteins were transferred overnight from the gel onto a PVDF membrane (Roche diagnostics, Meylan, France) prewetted in methanol and soaked in a blotting buffer. After blotting, the membrane was briefly washed with TBS, pH 7.5 (50 mM Tris base, 150 mM NaCl). Non-specific binding of antibody was blocked by incubating the membrane for 20 min in 1% (w/v) BSA, then 20 min in 1% (w/v) gelatin dissolved in TBST (TBS supplemented with 0.05% (v/v) of Tween-20). The membrane was then incubated during 2 hr at RT with the primary antibody diluted to 1:1000 in TBST. The membrane was washed four times for 10 min each in TBS, then incubated for 1 hr at RT with Alcaline phosphatase-labeled anti-rabbit-IgG-conjugated diluted in TBST. After washing the membrane as described above, proteins were revealed using the substrate BCIP/NBT (Sigma).

2.4. Cell culture

MLEC were cultured at 37° in a 5% CO_2 incubator with humidified atmosphere. Dulbecco's modified Eagle's medium (DMEM) with high glucose concentration (4500 mg/L) (Gibco BRL, Life Technology), with 5% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin sulfate (100 μg/mL) was used as the culture medium and was supplemented with 200 μg/mL of geneticin sulfate (G418 sulfate) (Gibco BRL) before use. The cells were passed for a maximum of 35 times.

2.5. TGFβ1 assay

TGFβ1 activity was assessed with a convenient and sensitive bioassay [25,30], making use of MLEC transfected with a PAI-1 promoter-luciferase construct (PAI/Luc).

MLEC's were trypsinized with trypsin-EDTA (Gibco BRL) and then seeded into 2 mL of DMEM/10% FCS/ antibiotics in a 6-wells tissue culture dish. Depending on the incubation time (4, 24 or 48 hr as mentioned herein), the cell density varied from 1×10^5 cells to 3×10^5. At the end of the incubation time, cells were subconfluent. They were rinsed twice with phosphate buffer saline (PBS) and the medium was replaced with the test sample in DMEM/ 0.1% (w/v) BSA containing TGFβ1 and/or polysaccharides. Incubation was done for 24 hr at 37°. Cells extracts were prepared and assayed for luciferase activity using the luciferase assay kit (Promega) as per the manufacturer's instructions. Briefly, the cells were washed twice in ice-cold PBS and then lysed with 150 μL of lysis buffer (Reporter lysis buffer, Promega). Dishes were scraped and cell lysates were centrifuged at 12,000 *g* for 15 s to pellet the cell debris. The supernatants were frozen and kept at −80°. Luminescence in relative light units (RLU) of 100 μL lysate from each well was measured in a Berthold Lumat 9501 for 10 s following the addition of 100 μL of subtrate (Luciferase assay reagent, Promega). Each experiment was performed twice and all assays were performed in triplicate. Error bars in figures correspond to the standard error of the mean of the samples. In parallel, two control wells from each plate were counted with a Coulter counter (Coultronics) in order to quantify the cells number.

3. Results

An array of dextran derivatives with a high degree of substitution in CM groups (between 0.6 and 1) and increasing degree of substitution in B groups (0.15–0.43) and/or Su groups (0.64–1.08) was used in this study and is presented in Table 1.

3.1. Complex formation between dextran derivatives and TGFβ1

To study the interaction of different dextran derivatives with TGFβ1, an affinity-coelectrophoretic technique (AEC) was used, based on a nondenaturing, nonreducing agarose electrophoresis system. In order to avoid any decrease in TGFβ/polymer interaction due to the labeling process [15], a nonradioactive method (Western blotting) was employed to detect the growth factor. Dextran derivatives are anionic due to the presence of carboxymethyl and sulfate moities. Thus, their electrophoretic mobility is much higher than the one of TGFβ1 at neutral pH. Consequently, the binding of the growth factor to these anionic polymers increased the TGFβ1 migration towards the anode.

Dextran derivatives with increasing benzylamide and sulfate substitution levels were evaluated using this technique to determine the impact of each chemical group on

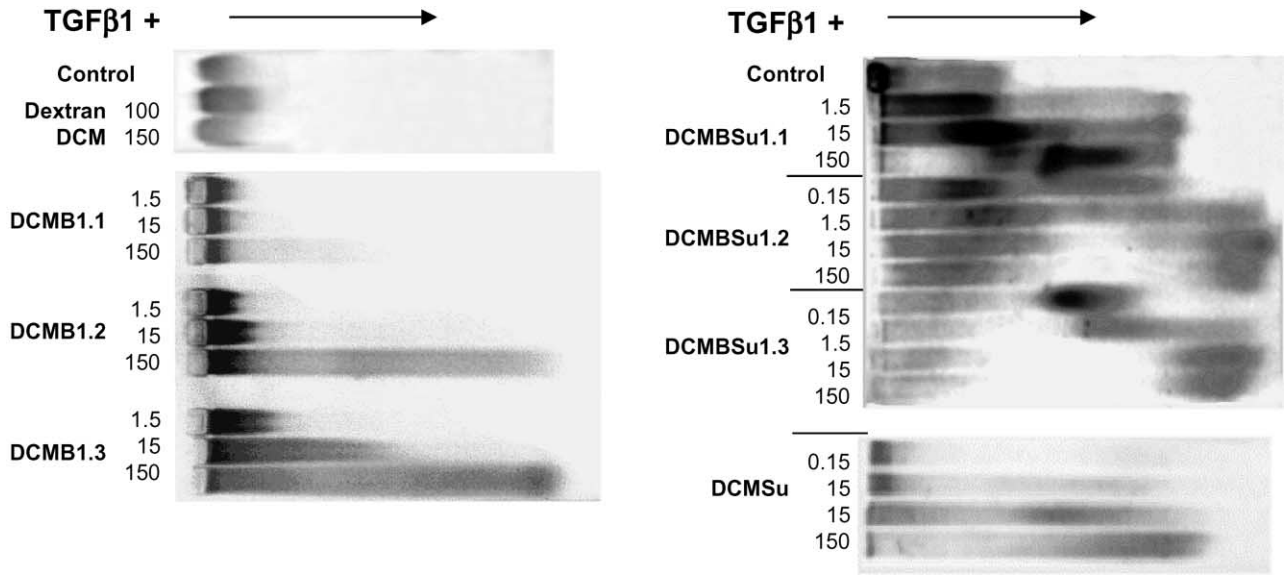

Fig. 1. Increased electrophoretic mobility of TGFβ1 by anionic dextran derivatives. TGFβ1 (60 nM; control) was mixed with native dextran, DCM, various DCMB with increasing contents of B groups, various DCMBSu with increasing contents of Su groups or DCMSu (polymer concentrations are in μM). Electrophoresis in a 0.8% agarose gel was performed in a nondenaturing, nonreducing agarose gel electrophoresis system. The growth factor was transferred onto a PVDF membrane then detected by Western blotting.

its interaction with the TGFβ1. In response to native dextran and carboxymethyl dextran (DCM), TGFβ1 mobility was unchanged. On the contrary, in the presence of DCMB, DCMBSu and DCMSu, the migration of TGFβ1 was increased towards the anode, demonstrating an interaction between the polymers and the growth factor. With enhanced polymer concentration, migration of the protein front was speeded up in a dose-dependent manner (Fig. 1). In most cases, the protein was detected as a smear. Nevertheless, the migrating TGFβ1 front was split in two by DCMBSu1.2 and DCMBSu1.3 into two distinct fronts, illustrating the presence of two major molecular populations, one with high affinity toward the TGFβ1 and the second with low affinity. It is worth noting that the proportion of these two DCMBSu fractions varied in favor of the high affinity fraction as the sulfate content increased.

Moreover, TGFβ1 migration increased when mixed with DCMB substituted with a high benzylamide content, suggesting that the TGFβ1–DCMB complex formation was improved with the benzylamide substitution. In the same way, DCM or DCMB functionalized with increasing sulfate groups content enhanced the electrophoretic mobility of TGFβ1. It appeared that benzylamide and sulfate groups were both necessary for an optimal interaction with the growth factor since it showed a larger migration with DCMBSu1.2 than it did with DCMSu in spite of their same sulfate content.

These data indicated that the optimal polymer interaction with TGFβ1 appeared to be a DCMBSu with a degree of benzylamide substitution of 0.35–0.4 (beyond this value, the polymer was too hydrophobic) and a degree of sulfate substitution above 0.5.

3.2. Effect of TGFβ1 on the induction of the PAI/Luc construct

In order to evaluate the biological relevance of this TGFβ1/polymer binding, a cell culture study was performed. In the first step, two parameters were investigated in order to define an optimal protocol. The studied parameters were the TGFβ1 concentration and the role of the plating time. Considering the above results which demonstrated that DCMBSu was the most suitable polymer in term of binding, a similar polymer (DCMBSu2) was used to determine the optimal culture parameters. These studies were performed in comparison with heparin, another polysaccharide known to interact with the TGFβ1 and potentiate its activity [15,17].

The use of MLEC transfected with a PAI/Luc construct provides a convenient and sensitive assay for quantification of bioactive TGFβ1. Indeed, Transforming growth factor usually exhibits bifunctional effects depending on cell types and culture conditions. Active TGFβ1 in the test sample binds to the receptors of the transfectants and induces PAI-1 expression, resulting in a dose-dependent increase in luciferase activity in the cell lysate. Using the standard protocol from Abe *et al.* [25] with a short plating time of 4 hr, serial dilutions of TGFβ1 induced the PAI/Luc construct with a dose-dependent fashion. A linear response was observed in the range between 0 and 0.2 ng/mL and a plateau was reached for growth factor concentrations above 0.5 ng/mL (Fig. 2A). It appeared that when the growth factor was mixed with 250 μg/mL of DCMBSu2 or heparin, a slight increase of biological response emerged for the lowest TGFβ1 doses.

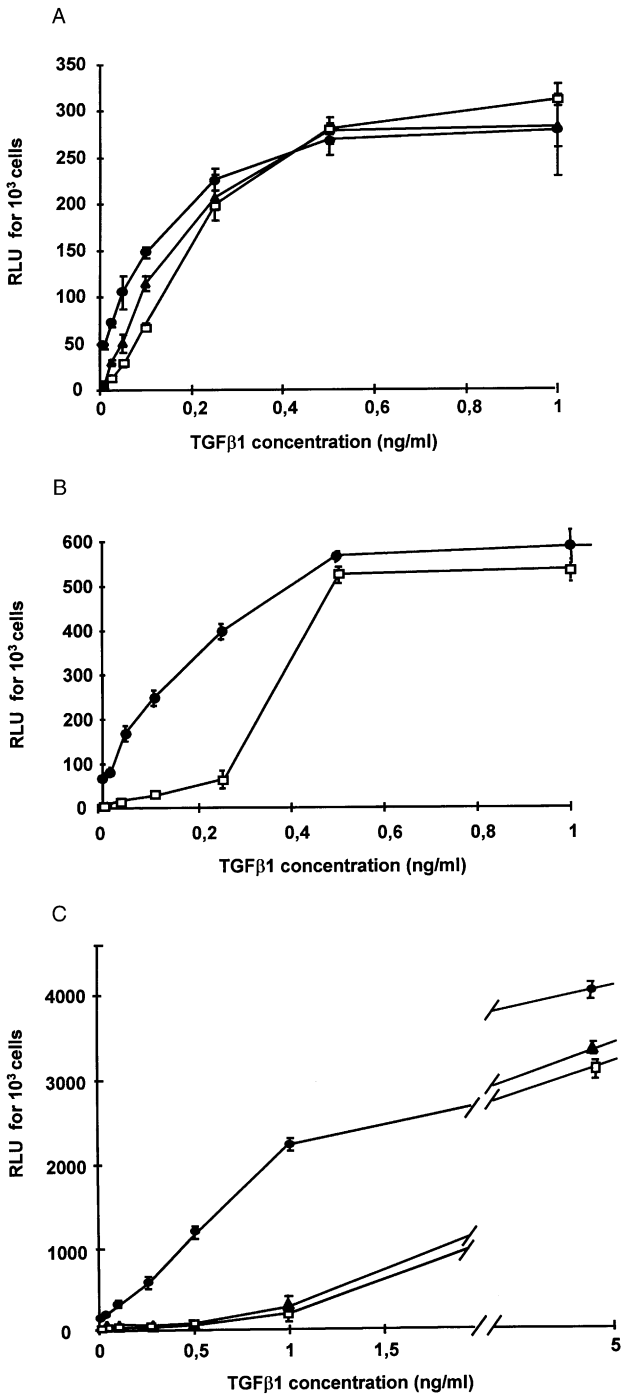

Fig. 2. Dose-dependent effect of TGFβ1 on the luciferase expression (RLU) and influence of the plating time. MLEC were allowed to attach and grow for 4 hr (A) 24 hr (B) or 48 hr (C) in DMEM supplemented with 10% of FCS prior the addition of samples. Samples containing increasing TGFβ1 concentrations in DMEM-0.1%BSA alone (□) or with 250 μg/mL of DCMBSu2 (●) or heparin (▲) were incubated with the cells for 24 hr prior to assaying for luciferase activity as described in Section 2.

However, the plating time seemed to affect this effect dramatically. The plating time represented the incubation time while cells grew in DMEM supplemented with 10% of FCS prior the addition of samples for 24 hr. Actually, when cells were previously grown for 24 or 48 hr, TGFβ1 biological effect on cells diminished dramatically (Fig. 2B and C). As the incubation time increased, the dose-dependent curve was shifted toward higher TGFβ1 concentrations. With a 48 hr plating time, the plateau was still not reached at the concentration of 5 ng/mL. Addition of 250 μg/mL of DCMBSu restored TGFβ1 biological effect shown by a strong enhancement of the luciferase gene reporter expression. When using a 0.25 ng/mL concentration of TGFβ1, a 48 hr plating time resulted in a 39-fold increase of luciferase expression while a 6-fold increase and only a 1.1-fold increase were observed with 24 and 4 hr plating time, respectively. In contrast, in these experimental conditions, the addition of 250 μg/mL of heparin with TGFβ1 did not modify the luciferase expression compared to the effect of TGFβ1 alone.

Considering these results, an intermediate plating time of 36 hr and a TGFβ1 concentration of 0.5 ng/mL were chosen to carry out the following experiments.

3.3. Screening of various dextran derivatives on the TGFβ1 biological response

The effects of native dextran and dextrans substituted with CM, CMB, CMBSu or CMSu were tested alone as control and mixed with TGFβ1 on MLEC's assay. Cells were previously incubated for 36 hr in the presence of 10% of FCS, and a dose-dependent effect of the polymers was studied (Fig. 3). In the absence of TGFβ1, 250 μg/mL of each polymer did not induce the PAI/Luc construct except the DCMSu which demonstrated a slight elevation of the luciferase activity. The association of TGFβ1 with native dextran or DCM did not modify its activity on the luciferase expression. In contrast, the induction of the PAI/Luc construct by TGFβ1 was amplified when mixed with DCMBSu2 and DCMB2 (15- and 6.5-fold increases respectively with the highest polymer concentration). These results indicated that polymers which did not exhibit any binding to the growth factor did not affect its biological response. Dextran and carboxymethylated dextran were totally inefficient in term of potentiation. On the contrary, benzylamide and benzylamide/sulfate substitution on the dextran backbone enhanced the growth factor biological response with a stronger effect with the second one. Surprisingly, DCMSu with the same sulfate content than DCMBSu2 increased only slightly the luciferase activity (2.5-fold increase) but the response was not dose dependent. This sulfated dextran demonstrated an effect on the luciferase expression similar to what was observed with the growth factor alone and its association with TGFβ1 likely gave rise to an additive effect on the luciferase expression.

3.4. TGFβ1 potentiating effect of active dextran derivatives through an early binding

These results showed that the DCMBSu2 was the most efficient polymer in term of TGFβ1 potentiation.

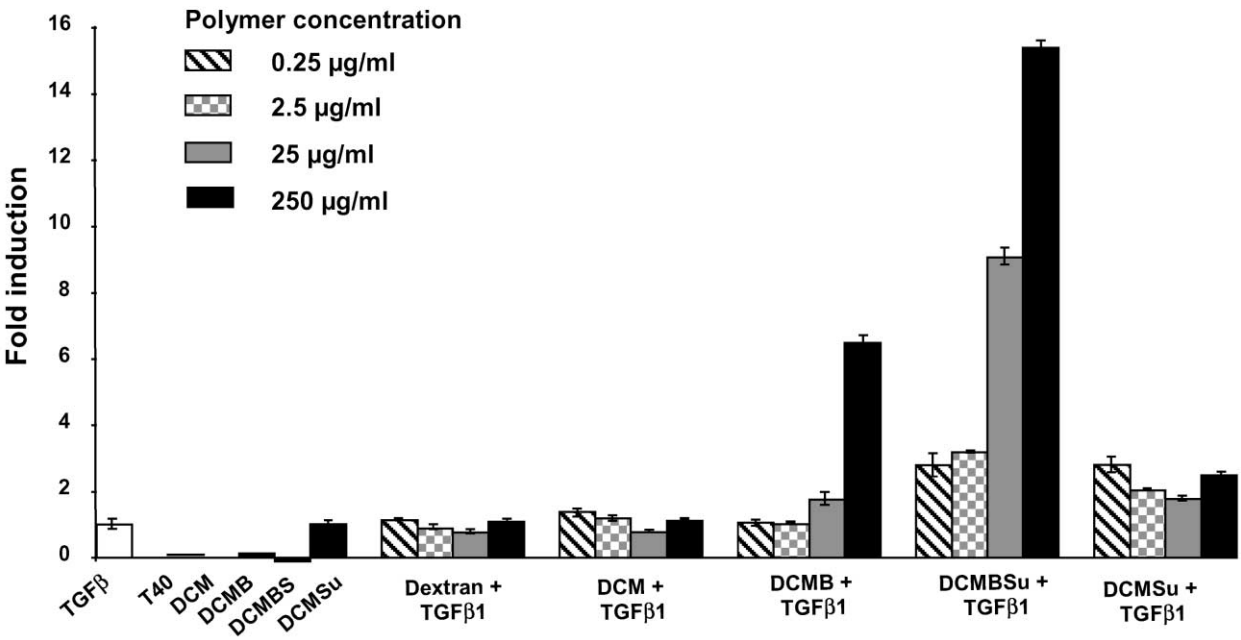

Fig. 3. Effects of dextran derivatives on the PAI/Luc and MLEC assay in the absence or presence of 0.5 ng/mL of TGFβ1 as indicated. Cells have grown for 36 hr in DMEM/10%FCS prior the addition of polymers with/without the growth factor in DMEM-0.1%BSA for 24 hr. The values represent the fold induction of the luciferase activity compared to that TGFβ1 control.

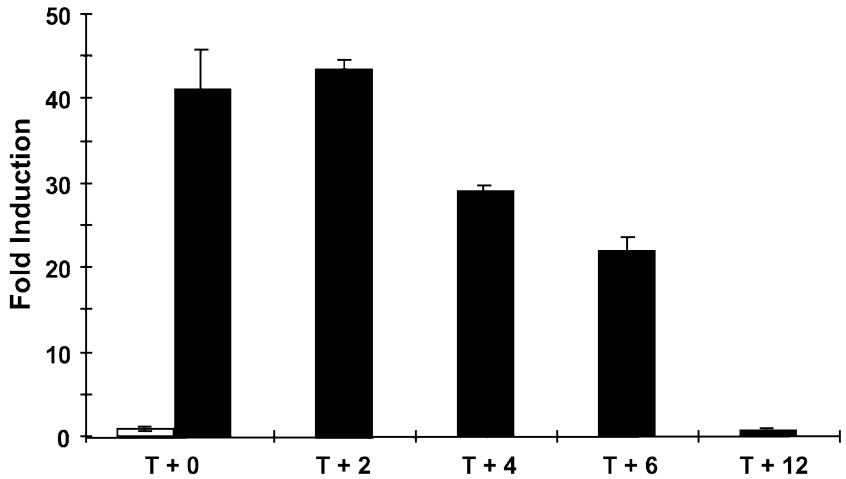

Fig. 4. Time effect of DCMBSu2 on the induction of the PAI/Luc by the TGFβ1. MLE cells were incubated for 36 hr in DMEM/10%FCS. These were then incubated for 24 hr in DMEM/0.1% BSA containing 0.5 ng/mL of TGFβ1 (control). DCMBSu2 (250 µg/mL) was added to cells at the indicated time after the addition of the growth factor. The values represent the fold induction of the luciferase activity compared to that TGFβ1 control.

However, one question remains: does this potentiation require a direct and early binding of both components or can it be achieved later on? As TGFβ1 can be regulated through multiple binding sites, one may wander if extracellular matrix components could interact with this growth factor, thus reducing functionalized dextrans potentiation.

DCMBSu2 was added to the culture medium with a delay of 0, 2, 4, 6 and 12 hr after the initial addition of 0.5 ng/mL of TGFβ1 (Fig. 4). It appeared that the potentiating effect of the DCMBSu2 was maximal when added simultaneously or 2 hr after the growth factor. However, when the polymer was added later than 2 hr, its potentiation on the TGFβ1 activity decreased with a time delay and was cancelled when added 12 hr later the growth factor. These results indicated that the potentiating effect required a complex formation between the polymers and TGFβ1 in an unbound form. Moreover, the polymer seemed unable to displace TGFβ1 from its other environmental binding molecules.

4. Discussion

The diverse biological actions of the proteins of TGFβ family suggest that they play a major role in physiology and pathology. It is likely that their activation, diffusion

and degradation processes are tightly controlled. The study indicates that some dextran derivatives may act as TGFβ1 regulatory factors. Our results support and extend a previous finding that a dextran substituted with only CM and B groups is capable to bind to TGFβ1 [22].

However, the present study is the first one that analyzes the effect of each substituent group (carboxymethyl, benzylamide and sulfate) on the interaction with the growth factor. It appears that carboxymethylated dextran do not interact with TGFβ1, supporting the concept that the presence of the other chemical groups is a prerequisite to achieve an adequate binding. From the binding shift assays in most cases, the protein was detected as a smear. Two hypotheses may explain this phenomenon: (i) the statistical nature of susbtitutions of the employed polymers may result in affinity variations of the various polymeric chains with the growth factor; (ii) each macromolecule could not migrate with the same rate due to the polymer polydispersity. The use of a polymeric fraction separated by steric exclusion chromatography with a polydispersity indice close to 1 would discriminate both effects. Nevertheless, the polymers still can be classified according to an index of affinity towards the growth factor: DCMBSu > DCMSu > DCMB⋙DCM = dextran. However, in each polymer group, the substituted dextran–TGFβ1 interaction is improved when increasing the benzylamide content (a degree of substitution around 0.35) as well as the sulfate content (a degree of substitution above 0.5). Thus, the compound demonstrating the best affinity for the growth factor contains both benzylamide and sulfate substitutions. Their effect on the TGFβ1 biological activity confirms this phenomenon. Only polymers able to interact with the growth factor exhibit also a TGFβ1 potentiating effect. It is worth noting that DCMSu demonstrates a much weaker activity on MLEC when associated with TGFβ1 than DCMBSu2, in spite of similar polymeric sulfate content.

TGFβ1 is known to bind to certain sulfated or phosphated polysaccharides such as heparin, heparan sulfate, dextran sulfate and fucoidan but not to related and equally charged agents such as chondroitin [15,17]. Polysaccharide structure studies demonstrate that N-sulfated glycosaminoglycans are required in order to obtain a TGFβ1 binding. In TGFβ, basic amino acid residues including lysines and arginines contribute to a strongly positively charged surface which allows an interaction with highly sulfated sequences. However, a specific binding is a complex phenomenon that requires more than an excess of a particular sulfate density [31]. In order to obtain an optimal interaction with the growth factor, our data suggests that benzylamide and sulfate susbtituents act synergistically. This observation confirms previous data showing that pure electrostatic interactions are insufficient to bind polysaccharides to proteins [32]. Efficient binding requires other interactions such as hydrophobic ones or secondary and tertiary protein structural integrity [33]. Recently, Bittoun *et al.* have shown that DCMB with (B) degree of substitution (d.s.) of 0.3 forms a stable 1:1 complex with FGF-2. As in this study, fluorescence anisotropy analysis indicated a K_d of 20 ± 10 nM, we hypothesized that suitable dextran derivatives interact with TGFβ1 with a K_d in the same range.

From the results obtained with DCMBSu (the most efficient polymer found in this study), it seems clear that the potentiating effect of these polymers on the TGFβ1 biological response is related to their ability to interact with this growth factor. When added 4 hr after the growth factor, the DCMBSu exhibits a reduced potentiating effect. This effect even disappears when the polymer is added 12 hr after TGFβ1. Thus, a direct and early binding is required to optimize TGFβ1 biological activity.

The potentiating effect is more significant when polymers are combined with the lower growth factor concentrations. For higher TGFβ1 concentrations, the biological response with or without polymers seems to reach the same plateau, indicating that the cells receptors have been saturated by an excess of growth factor. This result shows that DCMBSu does not act on the intracellular signalling pathway, but rather stabilizes and promotes the TGFβ1 signal in the pericellular environment. Furthermore, the plating time of MLEC before the addition of the growth factor seems to be an essential parameter. Two main hypotheses may explain this phenomenon:

(i) Entrappment and binding with extracellular matrix components. TGFβ has been shown to bind to the core proteins of at least two proteoglycans (betaglycan and decorin) [4,5,34]. In particular, decorin is known to interact with TGFβ, thereby inactivating the molecule [35–37].
(ii) One cannot exclude another inhibition process through cellular activity the production of which is time related and resulting in an enzymatic degradation or a factor inactivation.

Whatever the considered hypothesis, we can conclude that dextran derivatives compete with these processes keeping the growth factor available to interact with its cell membrane receptors. Our results indicate that the TGFβ1 is rapidly inactivated since the potentiating effect of DCMBSu2 decreases when the polymer is added to the medium 4 hr later than the growth factor.

As demonstrated with heparin and fucoidan [16], DCMBSu2 protected TGFβ1 from the enzymatic degradation (D.L.A. unpublished observations), thereby stabilizing the growth factor in an active conformation. This property of heparin and heparin-like polysaccharides was also shown with FGFs which thereby exhibited a potentiated mitogenic activity in many cells [23,24,38–40]. Further investigations have also shown an inhibition of cell proliferation by dextran derivatives in various cell lines as osteoblasts or tumoral epithelial cell lines [41–43]. The authors postulated that the polymers may act by trapping

growth factors and therefore displaced the binding of these growth factors to their high affinity membrane [22,33]. However, because TGFβ1 signaling on MLEC is amplified by the presence of DCMBSu2, we can exclude a trapping of the growth factor by the polymer. On the contrary, these functionalized polymers seemed to promote the binding of TGFβ1 to their cell surface receptors.

Interestingly, neither binding with TGFβ1 (data not shown), nor promoting effect on the TGFβ1 activity were observed in presence of heparin in our experiments. The inefficiency of heparin used in this study might be due to its low molecular weight (18 kDa) compared to the molecular weight of DCMBSu2 (90 kDa). However, some authors demonstrated that heparin/liver heparan sulfate potentiated TGFβ1 effect by acting as antagonists of serum α2-macroglobulin, a natural TGFβ ligand [31]. The potentiation of TGFβ1 in absence of serum by dextran derivatives but not by heparin, highlighted that these functionalized polymers might act as direct modulators of receptors binding/activation. Dextran derivatives and heparin seemed to act on the TGFβ1 signal using different pathways.

The evidence suggests two possible roles for the dextran derivatives binding in the modulation of TGFβ1 activity: (i) to localize and keep TGFβ1 in the extracellular space in an active form, by protecting TGFβ1 from extracellular and intracellular degradation proteolysis and (ii) to accumulate TGFβ1 in the pericellular environment, thereby enhancing its effect as a local growth/metabolic signal by chaperoning TGFβ1 to key extra/intracellular sites.

Selective interaction of a suitable functionalized dextran with TGFβ1 may have significant biological implications. TGFβ has three major biological effects—stimulation of extracellular matrix formation, growth inhibition, and immunosuppression. Future therapeutic TGFβ applications could be either used to enhance bone repair. Because exogenous active TGFβ1 is quickly cleared from the circulation with a half-life of 2.2 min [44], successful treatments of nonhealing bone defects with this growth factor are obtained with much higher concentrations than those present in normal tissues. To avoid undesired systemic effects, it may be more effective to employ TGFβ1 in combination with an appropriate protective compound. Though the precise cellular function of the TGFβ/dextran derivatives selective interaction is not yet completely defined, the association of these functionalized dextrans to the growth factor may have a significant biological interest for a potential therapeutic TGFβ1 use in wound healing.

The influence of dextran derivatives on TGFβ1 activity could explain some of their biological actions. These molecules were shown to stimulate the bone repair of trephine skull defects in rats [45,46]. This stimulation could be related to their ability to promote the differentiation of bone-forming cells, since they stimulated the expression of osteoblast features [42]. Nevertheless, the present study revealed that the ability of these polymers to bind TGFβ1 and to promote its activity may be useful for a practical application in the field of bone tissue repair.

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