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Inhibitory effect of substituted dextrans on MCF7 human breast cancer cell growth *in vitro*

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Substituted dextrans can reproduce some of the properties of heparin and can thus be used to alter cellular growth. We studied the effect of heparin (H108), dextran (D), carboxymethylbenzylamide dextran (CMDB) and carboxymethylbenzylamide sulfonate dextran (CMDBS) on the growth of human mammary cells of the MCF7 tumor line. The cells were cultured in minimum Eagle's medium containing 2% fetal calf serum without biopolymer, or with increasing concentrations of H108, D, CMDB or CMDBS. Growth curves were accurately based on cell counting using a Coulter counter. Cell distribution in the various phases of the cycle was analyzed by flow cytometry. Dose-dependent growth inhibitory effects (400–4000 μg/ml) were observed. The effect on MCF7 tumor cells was most apparent with CMDBS. The percentage of cells in the S phase decreased with preferential blocking in the G_0/G_1 phase. Pre-clinical studies can be anticipated as there is an absence of *in vivo* toxicity.

Key words: Cellular growth inhibition, heparin analog, mammary carcinoma

Introduction

Glycosaminoglycans such as heparin,[1,2] in addition to their well-known action on coagulation, possess effects which regulate cellular behavior. These molecules can thus influence blood vessel growth.[3] They can also modify cellular growth. Clowes *et al.*[4] were the first to demonstrate the inhibition of vascular smooth muscle cell proliferation by heparin. Paul *et al.*[5] studied the inhibition of 3T3 fibroblast growth and Hoover *et al.*[6] that of rat arterial muscle cells. Mesangial cells such as these are inhibited both *in vitro* and *in vivo*.[7] Certain epithelial cells are also sensitive to the inhibitory effect of heparin. This is the case with rat cervical epithelial cells,[8] and human and mouse keratinocytes.[9] More recently, Biswas[10] has shown the existence of heparin and heparin sulfate receptor sites on the surface of B16 melanoma cells. Halper and Carter[11] have studied the effect of heparin on SW-13 cells derived from a carcinoma of the human adrenocortex. They observed an inhibition, dependent on dose and cellular density, that was reversible and most pronounced in cells growing exponentially, in single layer cultures. Growth in semi-solid agar, which can correlate with *in vivo* tumorigenicity,[12] is also affected. Heparin and some analogs certainly interact with some growth factors or their receptors.[13,14]

Together with these natural compounds, several functional synthetic dextran polymers and cyclodextrins[15] are able to exert analogous effects on the processes implicated in tumor development. It seemed interesting to study the action of these molecules on tumors in the hope of identifying some therapeutic applications. We present the results here of the study on the modulation of growth of human mammary carcinoma cells (MCF7) by substituted dextrans.

Materials and methods

Characteristics of the MCF7 line

The MCF7 eptihelial cells are derived from a pleural effusion in a 69 year old female patient who had

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been treated with hormones and radiotherapy. The source is based on, and has been characterized by, the work of Soule *et al.*[16]

These cells retain certain characteristics of the mammary epithelium, such as the utilization of estradiol as a growth factor by a protein receptor intermediary, capable of self-fixation in the nucleus, in the presence of estradiol.

The whole study took place using passages 41–59 of the MCF7 source available from L'Institut d'Oncologie Cellulaire et Moléculaire Humaine de Bobigny.

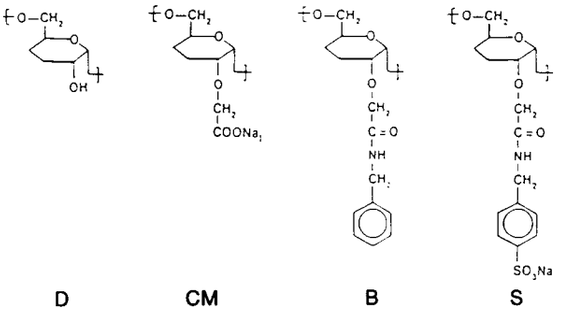


Figure 1. Structures of dextran (D), and carboxymethyl (CM), carboxymethylbenzylamide (B) and carboxymethylbenzylamide sulfonate (S) substitutions carried out to produce various derivatives.

Conditions of culture

The line is generally cultured in 75 cm^2 sterile plastic flasks (T75 Falcon, Gibco) in an incubator at 37°C (5% CO_2, 95% air) in the presence of minimum Eagle's medium (MEM) containing 5% sodium pyruvate (Gibco sodium pyruvate 7.5%), 10% antibiotics (penicillin and streptomycin) and 10% fetal calf serum (FCS). Each week the cells are sub-cultured after detachment with a solution of trypsin–EDTA (0.02%); usually one flask into two flasks.

The whole of the study was carried out with cultures of MEM enriched with 2% FCS. This low level of serum was chosen to reduce the effect of serum growth factors on the cultured cells. However, it allows satisfactory and rapid growth.

Production of substituted dextrans

Water soluble dextran derivatives were prepared from dextran T40 (D; Pharmacia Fine Chemicals, Uppsala, Sweden).

Dextran [$DX(OH_3)$] was first carboxymethylated using a step-by-step procedure (using three steps in water at 50°C for 40 min; molar ratio $ClCH_2COOH/DX(OH)_3 = 3.5$). Benzylamine was coupled to methycarboxylic dextran in the presence of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydriquinoline (EEDQ), and the sample was then precipited in methanol, washed and dried under vacuum. Two successive sulfonations of the aromatic ring of benzylamine were achieved by adding chlorosulfonic acid in dichloromethane (150 mM). A range of dextran derivatives, with various proportions of the substitutions shown in Figure 1, was produced. These derivatives were then purified and characterized according to the percentage of substituted functions. These molecules demonstrate anti-coagulant effects which vary according to their composition (Table 1). The heparin (H108) used came from the Institut Choay.

Growth curves

On day 0, MCF7 cells were plated at a density of 2×10^4 cells/well in 24-well tissue culture plates in DMEM with 2% FCS. At 24 h later growth medium was changed for medium containing H108, CMDB or CMDBS. On certain days thereafter, cells were trypsinized, harvested and counted in a Coulter counter.

Flow cytometry

The distribution of cells in the different phases of the cell cycle can be assessed by flow cytometry after the DNA has been marked with prodidium iodide. The cells are detached with trypsin–EDTA, rinsed

Table 1. Characterization of substituted dextrans

	Substitution (%)[a]				Anticoagulant activity (UI/mg)
	D	CM	B	S	
T 40	100	0	0	0	0
H 108	0	0	0	0	173
CMD6B	0	59	8	0	0
CMD2BS	6	76	0	14	3.2
CMD9BS	0	74	16	10	5.2
CMDBS6	37	41	0	22	—
CMDBS-NH_2	0	58	19	26	—

[a] D = dextran; CM = carboxymethyl; B = carboxymethylbenzylamine; S = carboxymethylbenzylamide sulfonate. (See Figure 1 for structures of substituted groups.)

twice in PBS buffer and resuspended at a concentration of 1×10^6 cells/ml. After centrifuging, the washed sediment was agitated vigorously in 1 ml of propidium iodide, then incubated for 10 min at 37°C. This was followed by the addition of 100 µl of 9% Nacl. The cells were then analyzed by flow cytometry. The percentage of cells in the G_0/G_1, S and $G_2 + M$ phases of the cycle was obtained from histograms where the abscissa represents the amount of fluorescence, proportional to the amount of DNA in each cell, and the ordinate the number of cells. Cytofluorometry, in addition, allows analysis of cellular size and density of granulations. Only the dextrans and the concentrations which appeared to have the most marked effect on proliferation were tested. These were CMDBS6 and CMBD-NH_2.

Results

Growth curves

With heparin (H108), some disturbance of growth is noted at concentrations from 40 to 400 µg/ml. A slowing down in growth is, however, apparent with a concentration of 4000 µg/ml. The curves obtained with CMD6B have a fairly similar appearance. Of more interest is the study of the curves using dextrans with more substitutions, particularly the introduction of sulfonate groups. Low (40 µg/ml) and moderate (400 µg/ml) concentrations only show a mild disturbance of cell growth (Figure 2). Conversely, at a concentration of 4000 µg/ml, a cytotoxic effect is noted with compound CMDBS6. This effect is not, however, definitive as there is an increase in the curves after the sixth day, indicating a resumption of proliferation from the viable cells. For an intermediate concentration (1000 µg/ml), a flattening of the curve is observed, which suggests a cytostatic action involving CMDBS6 and CMDBS-NH_2.

Percentage of inhibition

Table 2 is a résumé of the percentages of inhibition measured 6 days after the addition of various polymers. From Table 2 it is observed that

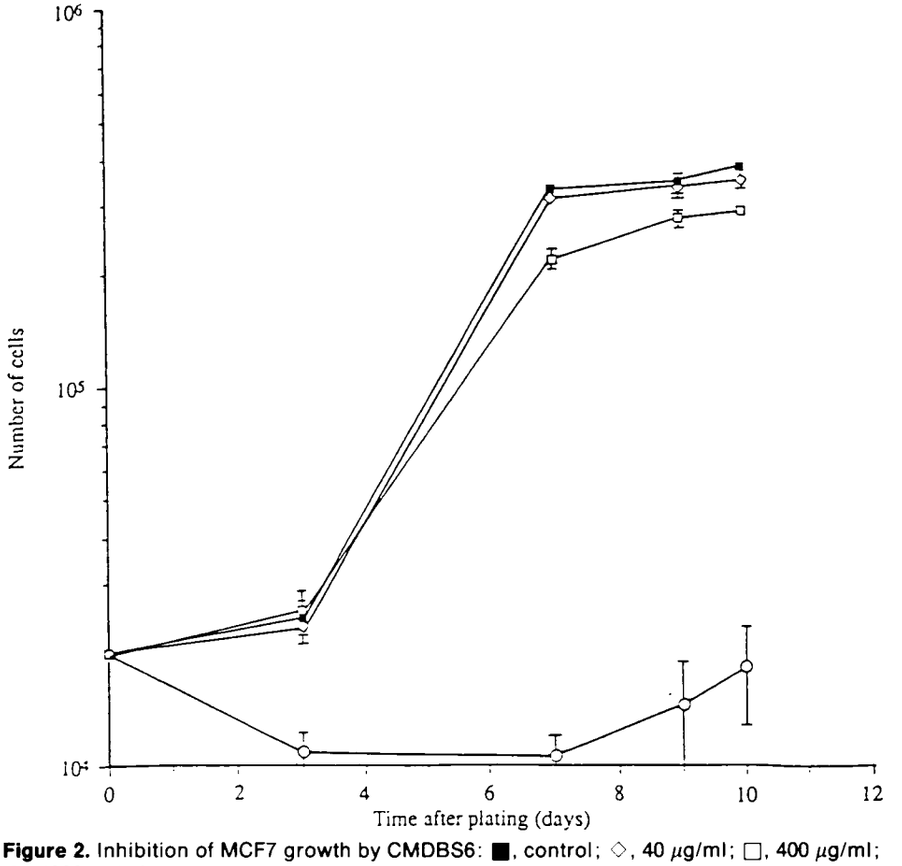


Figure 2. Inhibition of MCF7 growth by CMDBS6: ■, control; ◇, 40 µg/ml; □, 400 µg/ml; ○, 4000 µg/ml.

Table 2. Doubling time (h)

	Concentration (μg/ml)				
	0	40	400	1000	4000
H 108	33	34	36	—	46
CMDB	33	36	33	—	44
CMDBS6	37	41	46	45	97
CMDBS-NH_2	33	—	34	37	67

heparin (H108) and CMD6B have similar effects. At low concentrations (40 μg/ml) they produce a slight stimulation of proliferation. The inhibitory effect only becomes apparent at higher concentrations (4000 μg/ml). The sulfonate derivatives have an inhibitory capacity which is more marked at high concentrations; however, this effect is already apparent at concentrations four times lower than those of the previous compounds. The growth inhibition curve, as a function of the concentration of dextran, has been plotted for CMD6B-NH_2. It shows a regular dose–effect relationship.

Doubling time

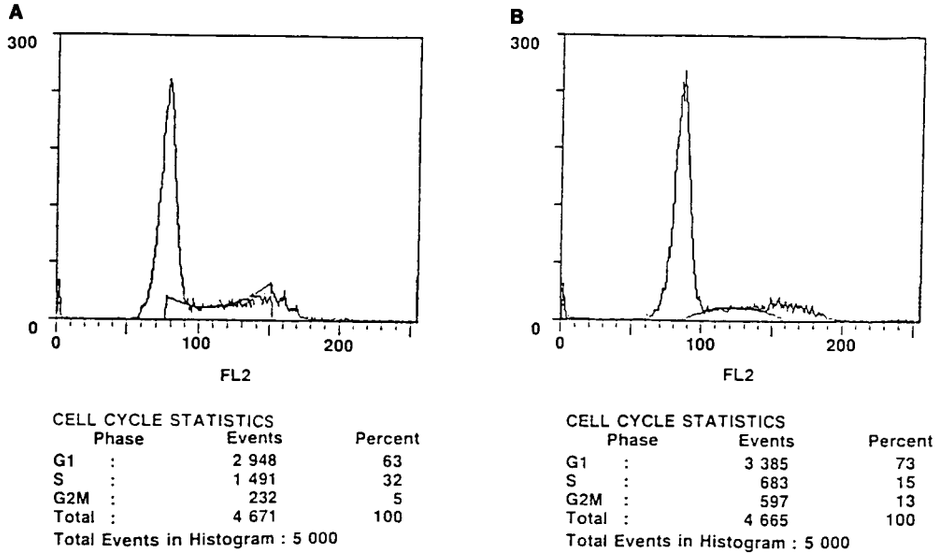
The different products tested show an increase in doubling time observed before the plateau. This increase is very pronounced for concentrations of 4000 μg/ml. Only CMDBS6 shows an increase in doubling time at a concentration four times lower (Table 3).

Analysis of the cell cycle by cytofluorimetry (Figure 3)

The population of MCF7 cells in the exponential growth phase shows a distribution in the cell cycle

Table 3. Inhibition of monolayer growth of MCF7 by substituted dextrans

	Concentration (μg/ml)			
	40	400	1000	4000
H 108	+3%	12%	—	−52%
CMD6B	+8.5% (+0.7%)	−5% (+5%)	−16%	−59% (±19%)
CMD2BS	−15%	—	—	−99%
CMD9BS	—	—	—	−93%
CMDBS6	−8% (±2%)	−24.6% (±11%)	−28.5% (±10%)	−95.5% (±2%)
CMDBS-NH_2	−12.5% (±4%)	−14.2% (±5%)	−32.5% (±17%)	−92%



CELL CYCLE STATISTICS

Phase	Events	Percent
G1 :	2 948	63
S :	1 491	32
G2M :	232	5
Total :	4 671	100

Total Events in Histogram : 5 000

CELL CYCLE STATISTICS

Phase	Events	Percent
G1 :	3 385	73
S :	683	15
G2M :	597	13
Total :	4 665	100

Total Events in Histogram : 5 000

Figure 3. DNA cell cycle analysis of (A) control and (B) CMDBS-NH_2-exposed cells.

of 50–60% for the G_0/G_1 phase, 25–30% for S and 5–7% for $G_2 + M$. After 48 h exposure to CMDBS-NH_2, an increase in the G_0/G_1 phase (73%) is observed compared with the non-treated control (63%). A decrease of 15% is also observed in the S phase as opposed to 32% in the controls.

This trend is again found after 48 h exposure to CMDBS6. A 78% increase in the percentage of cells in the G_0/G_1 phase is observed (controls 67%) as well as a 14% decrease in the percentage of cells in the S phase (control 25%). This build up of cells in the G_0/G_1 phase indicates a blocking in the G_1 phase and accounts, in part, for the inhibition of proliferation.

The percentage of cells in the $G_2 + M$ phase remains stable after exposure to CMDBS6 (8% as opposed to 7% control) and it rises slightly to 13% (control 5%) after exposure to CMDBS-NH_2.

It can be noted that if there were to be a single point of blocking in G_1, a phase in which cells are accumulating, then the percentage of cells in the $G_2 + M$ phase should decrease, as in the S phase. No such decrease is observed. It appears, therefore, that another point of blocking in the G_2 phase must exist. The analysis which occurs after 4 days of exposure to CMDBS-NH_2 does not, however, allow identification of the block in G_1. An increase in the percentage of cells in $G_2 + M$ is observed, which could be indicative either of an accumulation in the G_2 phase or a resumption of cellular proliferation.

Analysis of the size and density of granulation of cells by cytoflourimetry

The morphological study does not reveal any significant differences in size between controls and treated cell populations. However, the study of the granulation density revealed that treated cells are more granular than control cells.

Discussion

In the light of the results presented, several points merit discussion.

Heparin and the substituted dextrans have a negative modulation effect on the *in vitro* growth of MCF7 cells. This effect, obtained with epithelial tumor cells, confirms the results obtained by other authors with fibroblasts and muscle cells.[4–6]

Carboxymethylbenzylamide dextran demonstrates a similar activity to that of heparin. An inhibitory effect only appears at high concentrations (4000 μg/ml). The sulfonated dextrans demonstrate an increased inhibitory capacity. The inhibition begins at a concentration of 1000 μg/ml and is very pronounced at a concentration of 4000 μg/ml.

The mechanism of action of the dextrans is certainly not completely clear. It is probably not a one-to-one basis. The rapid fall in the lower part of the growth curves supports the concept of a cytotoxic effect at high concentrations. Analysis of the growth curves for lower concentrations and a study of the cellular cycle suggests a cytostatic effect.

The point of blocking in G_0/G_1 could be unique, with the pool of cells in G_1 eventually regulating that of the cells in G_2. A double blocking in G_0/G_1 and $G_2 + M$ could also exist.

The cytostatic effect could be non-specific. It may be secondary to a titration phenomenon of certain growth factors by the dextrans. Tardieu *et al.*[17] has shown an interaction between polymers and the fibroblast growth factor. However, there is a potentiation of the mitogenic effect which has been observed in this case. By virtue of their structure these polymers could also impede the access of certain of these growth factors to their receptor sites.

The cytostatic effect could also be the result of a direct effect of the polymers on the cell. These products could compete with the proteoglycans of the extracellular matrix. It is known that heparin competes with heparin sulfate at the level of its receptor site, and thus its reabsorption and nuclear level is diminished. For Conrad[18] this decrease in the nuclear level correlates with a lengthening of the cellular doubling time.

Finally, along with the effects on proliferation, a positive effect on differentiation can also be anticipated. This concept[19] is supported by the increase in the density of granulation observed with sulfonated dextrans.

In conclusion, this study demonstrated an *in vitro* negative modulation of the growth of human mammary tumor cells by substituted dextrans. The intimate mechanism involved in the inhibition of proliferation is not totally clear, but appears to be original. Confirmation involving other examples is underway.[20] Further preclinical studies are warranted since their anticoagulant activity is low.

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