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Effect of hyperthermia on the extracellular matrix

I. Heat enhances hyaluronan and inhibits sulphated glycosaminoglycan synthesis

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The synthesis of sulphated glycosaminoglycans (GAG) and hyaluronan (HA) was studied in 3 cell strains incubated at 37°C or 42°C. Cells were labelled with [^{3}H]glucosamine and [^{35}S]sulphate. No incorporation of [^{35}S]sulphate was observed at 42°C. Cellulose acetate electrophoresis of GAGs synthetized at 42°C showed only one [^{3}H]glucosamine-labelled band completely digested with *Streptomyces* hyaluronidase. The quantification of [^{3}H]glucosamine-labelled GAGs before and after hyaluronidase treatment indicated an increased rate of HA synthesis at 42°C as compared to the rate of synthesis at 37°C. These results suggest that hyperthermia stimulates HA synthesis and inhibits sulphated GAG synthesis in several cell strains which in turn may contribute to the modification of extracellular environment in inflammation.

Hyperthermia; Heat shock; Glycosaminoglycan; Hyaluronan; Inflammation

1. INTRODUCTION

Inflammatory phenomena generate a local and systemic increase of temperature [1]. The metabolic behaviour of cells in hyperthermia is fundamentally changed. In vitro, at temperatures above 41°C mammalian cells switch off the synthesis of all the proteins except for a few proteins named stress or heat shock proteins [2]. The information about temperature-dependent metabolism of extracellular matrix macromolecules is scanty. In many pathological conditions e.g. rheumatoid arthritis, local inflammation increases the temperature up to 4.5 degrees above the normal value [3]. Human synovial cells in vitro enhanced hyaluronan (HA, formerly hyaluronic acid) synthesis with increasing culture temperature [4]. In the present study we compared the synthesis of hyaluronan and sulphated glycosaminoglycans (GAG) in three strains of cells incubated at 42°C, the temperature which induced a massive synthesis of heat shock proteins [5].

2. MATERIALS AND METHODS

2.1. *Materials*

Dulbecco's modified Eagle's medium (DMEM), M 199 medium, foetal calf serum (FCS) and chicken embryo extract were purchased from Gibco BRL (Cergy Pontoise, France). D-[^{3}H]Glucosamine (specific activity 74 TBq/mmol) and [^{35}S]sulphate (specific activity 270 MBq/mmol) were purchased from Amersham (Les Ullis, France). Pronase (type IV from *Streptomyces griseus*) was from Sigma Chemical Co. (Saint Lous, MO, USA). Hyaluronidase (EC 4.2.2.1, *Streptomyces hyaluroniticus*, 100 turbidity U/mg) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Sulphate-free DMEM was prepared by Institut Jacques Boy (Reims, France).

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2.2. *Cell cultures*

Human foreskin fibroblasts were explanted and grown in DMEM containing 10% (v/v) FCS, 50 U/ml penicillin, 50 μg/ml streptomycin according to routine techniques. Confluent cultures between 4th and 8th passage were used. The rat myogenic cell line L6, purchased from ECACC (Porton Down, Salisbury, UK) was grown in DMEM containing 10% (v/v) FCS, 2% (v/v) chicken embryo extract and antibiotics as above. Human endothelial cells isolated from umbilical cord veins (HUVEC) as previously described [6] were cultured in M 199 medium containing 20% (v/v) FCS, the antibiotics, 50 ng/ml basic fibroblast growth factor, 50 μg/ml heparin. The cells were identified by their morphology and the presence of factor VIII. The 4th passage cultures were used.

2.3. *Metabolic labelling*

All cell cultures, were plated in Costar 6 well plates (10 cm^2) and used at confluency. The medium was changed into sulfate-free DMEM containing 4% dialyzed FCS. One group was preincubated for 30 min at 42°C. For metabolic labelling series of 4 wells were incubated for 10 h with 10 μCi/ml (0.185 MBq/ml) [^{3}H]glucosamine and 2 μCi/ml (74 KBq/ml) [^{35}S]sulphate. Control wells were incubated at 37°C and wells preincubated at 42°C were followed by incubation at the same temperature. All incubations were performed in a 95% air 5% CO_2 atmosphere.

2.4. *Glycosaminoglycan analysis*

The culture medium and the cell layer were collected separately. Medium was harvested and the cell layer washed 3 times with cold (4°C) phosphate-buffered saline. Medium was heated for 5 min at 100°C. The cell layer was dissolved in 0.1 M NaOH. Aliquots were taken for protein measurements [8]. The pH of the remaining part was adjusted to 8.0 with 2 M acetic acid. The extracts were digested for 24 h at 48°C with 0.4 mg/ml pronase in 0.05 M Tris-HCl, pH 8.0, 0.02 M $CaCl_2$ [7]. Pronase was precipitated with 10% trichloracetic acid and the supernatant was exhaustively dialysed against distilled water at 4°C. An aliquot was taken for the measurement of radioactivity and the remaining part was lyophilized.

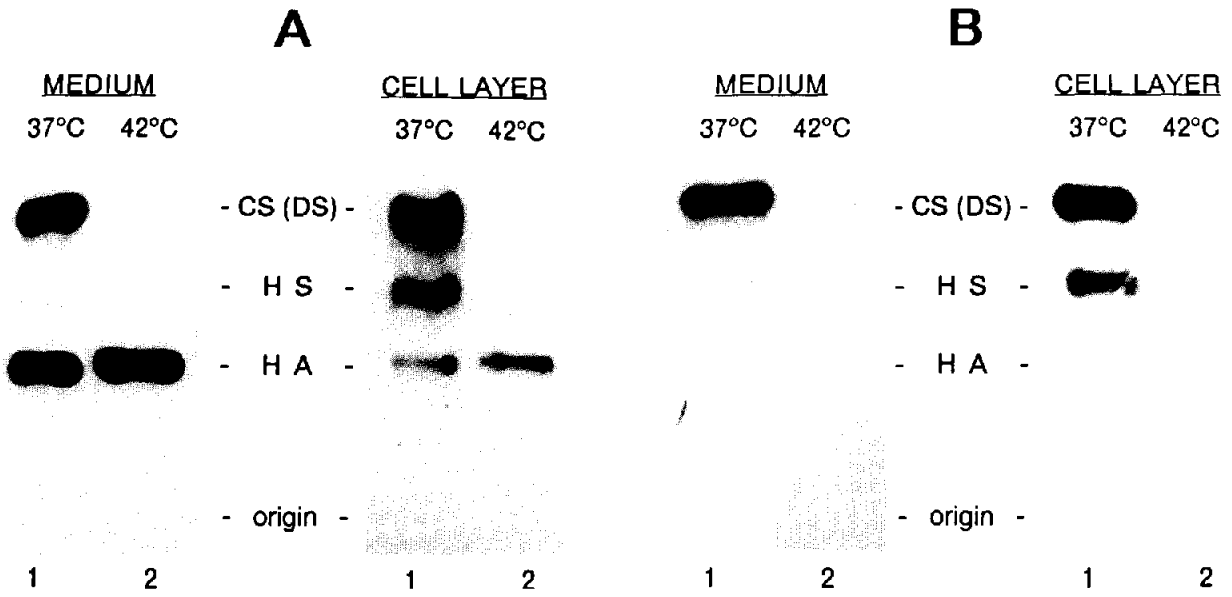

Fig. 1. Electrophoresis of GAGs synthetized by human skin fibroblasts before (A) and after (B) hyaluronidase treatment. Cells were incubated at 37°C (1) or at 42°C (2). GAGs from 5×10^5 cells secreted into the culture medium (left panel) and associated with the cell layer (right panel) were separated on Cellogel strips as described in section 2. After fixation and staining autoradiography was performed. The migration positions of hyaluronan (HA), heparan sulphate (HS) and chondroitin, dermatan sulphate (CS) standards are indicated by thin bars. Origin = application point.

Lyophilized samples were fractionated by cellulose acetate gel electrophoresis on Cellogel membranes (Chemetron, Milan, Italy). An aliquot of radiolabelled GAGs was applied on the membrane and the electrophoresis was performed in 0.1 M HCl at 40 V for 2 h [9]. Standard GAGs were deposited beside the studied samples in order to identify the position of the separated bands after migration. The membranes were then fixed in absolute ethanol, stained with 0.2% (m/v) Alcian blue and soaked with 1% (m/v) PPO, 5% (v/v) glycerol in ethanol. The dried strips were autoradiographied at −80°C on hyperfilm-MP (Amersham) in a X-omatic cassette with intensifying screen (Kodak).

To quantify hyaluronan synthesis aliquots of the pronase digests were hydrolysed at 60°C for 60 min with 10 U/ml hyaluronidase [10]. The samples were then spotted on 3 MM Whatman filter papier and dialysed against four changes of 1% (m/v) cetylpyridinium chloride [11]. The remaining radioactivity of the precipitate was counted in a liquid scintillation counter.

3. RESULTS

To study the effect of hyperthermia on GAG synthesis confluent dermal fibroblasts were labelled for 10 h with [^{35}S]sulphate and [^{3}H]glucosamine at 37°C (control group) or at 42°C. After incubation over 80% of cells were viable by Trypan blue test. No changes in total protein content were observed. Cells incubated at 37°C incorporated both radiolabelled precursors into GAGs secreted into culture medium and those associated with the cell layer (Table I). Cellogel electrophoresis of culture medium GAGs in 0.1 M HCl allowed for the separation of two major fractions which migrated with HA and chondroitin (dermatan) sulphate (CS) standards,

Table I

Effect of hyperthermia on incorporation of [^{3}H]glucosamine and [^{35}S]sulphate into fibroblast glycosaminoglycans

Incubation temperature		37°C (cpm/ng total cell proteins)		42°C (cpm/ng total cell proteins)	
Hyaluronidase digestion		−	+	−	+
Culture medium	[^{3}H]	1908.5 ± 248.3	1124.1 ± 131.4	1082.7 ± 202.6	59.0 ± 15.7
	[^{35}S]	340.0 ± 32.4	331.3 ± 40.4	N.D.	N.D.
Cell layer	[^{3}H]	1121.3 ± 149.5	883.8 ± 197.3	300.7 ± 50.2	N.D.
	[^{35}S]	169.2 ± 31.6	157.3 ± 41.8	N.D.	N.D.

Cells were incubated at 37°C (control) or 42°C (hyperthermia) during metabolic test. Isolated GAGs were precipitated with 1% cetylpyridinium chloride [11] before (−) and after (+) digestion with hyaluronidase. Every data is the mean ± S.D. of 4 determinations. N.D. = not detected.

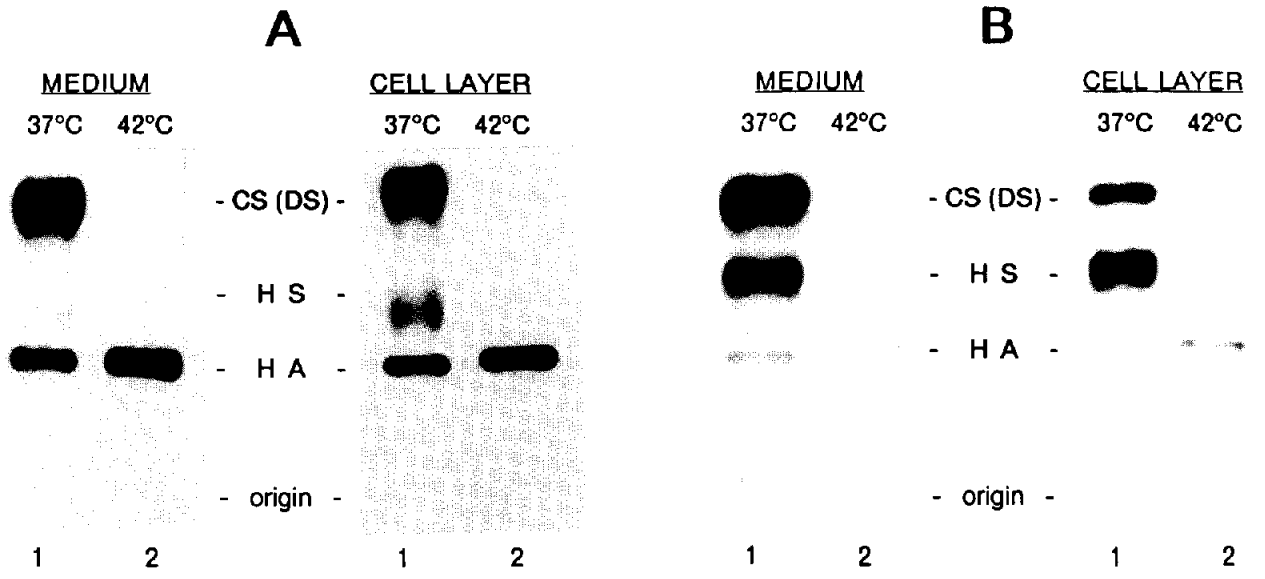

Fig. 2. Electrophoresis of GAGs synthetized by rat L6 myoblasts (A) and human umbilical cord endothelial cells (B). Cells were incubated at 37°C (1) or at 42°C (2). Other conditions are the same as in Fig. 1.

respectively (Fig. 1A, left panel). Cell-associated GAGs were separated on three fractions: HA, heparan sulphate (HS) and CS (Fig. 1A, right panel). The identification of the HA fraction was also done by digestion with streptomyces hyaluronidase (Fig. 1B). The bands that corresponded to the migration position of the HA standard were completely digested with the enzyme. When the cells were incubated at 42°C, no incorporation of [^{35}S]sulphate into GAGs was observed (Table I). Cellogel electrophoresis showed that only one major fraction migrated with the HA standard (Fig. 1A). This fraction was completely digested with hyaluronidase (Fig. 1B). About 40% of glucosamine was incorporated into culture medium HA at 37°C and over 95% at 42°C. Moreover, at 42°C the incorporation of [^{3}H]glucosamine into HA was enhanced by about 40% over that incorporated at 37°C. Those results indicate that HA is predominantly GAG-synthetized by dermal fibroblasts at 42°C and that HA synthesis is enhanced at this temperature.

To determine how general the effect of temperature might be, the GAG synthesis was investigated in L6 rat skeletal muscle myoblasts and HUVEC human umbilical cord endohelial cells. L6 myoblasts showed a similar pattern of GAG synthesis to fibroblasts (Fig. 2A). Cells incubated at 42°C did not synthetize sulphated GAGs. The total synthesis of HA increased more than twofold as judged by the difference of [^{3}H]glucosamine incorporation before and after hyaluronidase treatment (Table II). HUVEC synthetize a small quantity of HA at 37°C (Fig. 2B) as compared to other cells. This is in accordance with the published data [12]. Incubation of the cells at 42°C completely inhibited sulfated GAG synthesis but not HA synthesis (Table II). At 42°C most of HA was retained in the cell layer. Taken together the results indicate that different mammalian cells at hyperthermia

Table II

Effect of hyperthermia on incorporation of [^{3}H]glucosamine into glycosaminoglycans of L6 myoblasts and endothelial cells (HUVEC)

Incubation temperature	37°C (cpm/ng total cell proteins)		42°C (cpm/ng total cell proteins)	
Hyaluronidase digestion	−	+	−	+
L6 myoblasts				
Culture medium	1285.5 ± 99.7	893.3 ± 192.9	996.3 ± 53.2	N.D.
Cell layer	1914.0 ± 555.0	1565.0 ± 306.7	511.7 ± 83.7	N.D.
HUVEC				
Culture medium	1081.5 ± 122.7	945.2 ± 154.0	61.8 ± 6.9	N.D.
Cell layer	508.2 ± 73.8	409.3 ± 84.4	171.3 ± 70.6	N.D.

For explanation see legend to Table I.

conditions inhibit sulphated GAG synthesis and enhance hyaluronan synthesis.

4. DISCUSSION

Hyperthermia is a common phenomenon in inflammatory reaction and has a deep effect on the immune system and cell behaviour [1,13]. In the present study we showed that at 42°C in vitro several cell lines stopped sulphated GAG production and enhanced hyaluronan synthesis. This temperature was chosen because it is the highest temperature for the long survival of cells [14] as well as the lowest which induces large scale production of only heat-shock proteins [5].

Sulphated GAG are part of the proteoglycans. They are synthesized in the Golgi apparatus. The inhibition of sulphated GAG synthesis reflects the destruction of polyribosomes and protein synthesis machinery reported by several authors (for review see [14]). Synthesis of HA follows another pathway. The transferases of HA synthesis are located on the inner side of cell membrane and new chains can be started during ongoing synthesis [16]. The synthesis of HA may be enhanced because of: (i) a temperature effect on the catalytic process, (ii) an increase is the fluidity of the plasma membrane, (iii) a possible local increase in substrates which are not used in sulphated GAG synthesis.

Temperatures used to trigger the heat shock response in vitro are close to those attained in acute inflammation [15]. This process virtually induces deep changes in the phenotypic expression of many cell types in inflammed areas. Some of these changes are mediated by lymphokines [5]. In this report we showed that hyperthermia per se may also modify the expression of extracellular matrix macromolecules which in turn may change the cellular environment.

The intracellular protein synthesis during hyperthermia or heat shock has been extensively studied. Almost nothing is known about the effects of hyperthermia on the extracellular compartment. In many pathological processes accompanied by hyperthermia the extracellular matrix undergoes structural changes [15]. Moreover, it is now well established that matrix molecules influence cells. The roles of hyaluronan in many cell functions, particularly in the inflammatory response was largely studied [17]. Our results show dramatic changes in the expression of sulphated GAGs and HA under hyperthermia, and confirm the primordial role of HA in inflammation and suggest local deep changes in matrix composition. Further studies are needed to characterize the molecular mechanisms of heat influence on the extracellular matrix.

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