Heat shock of human synovial and dermal fibroblasts induces delayed up-regulation of collagenase-gene expression

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We investigated the effect of heat shock on the expression of the collagenase gene in normal human synovial and dermal fibroblasts. Heat shock (42–44 °C for 1 h) caused a marked increase in heat-shock protein 70 (HSP-70) mRNA levels, followed by a delayed increase in collagenase mRNA levels, in both cell types. Pretreatment with cycloheximide had no effect on the heat-shock-induced increase in HSP-70 mRNA expression, but abrogated the induction of collagenase mRNA during the recovery. To study the mechanisms of collagenase-gene induction by heat shock, the transcriptional activity of a collagenase-promoter-driven chloramphenicol acetyltransferase (CAT) reporter gene was examined in transient transfection experiments. Heat shock was followed by a > 2-fold increase in CAT activity driven by a

3.8 kb fragment of the collagenase promoter, or by a construct containing an AP-1 binding site. A mutation in the AP-1 binding site abolished the effect of heat shock. Electrophoretic-mobility-shift assays revealed a marked increase in DNA-binding activity specific for the AP-1 binding site in nuclear extracts prepared from synovial fibroblasts recovering from heat shock. These results indicate that heat shock causes a delayed increase in collagenase-gene expression in human fibroblasts, and suggests that this stimulation involves, at least in part, transcriptional activation through an AP-1 binding site. Heat shock appears to initiate a programme of cellular events resulting in collagenase-gene expression, and therefore may contribute to connective-tissue degradation in disease states.

INTRODUCTION

Matrix metalloproteinases (MMPs) comprise a group of enzymes responsible for the degradation of extracellular-matrix components. These enzymes are active at neutral pH, contain Zn^{2+} , and require Ca^{2+} for their activity (for reviews, see [1,2]). Collagenase (MMP-1), the prototypic member of this family, has the singular ability to initiate breakdown of native interstitial collagens (types I, II and III). Stromelysin (MMP-3) degrades the basement-membrane type IV collagen, as well as several non-collagenous components of the extracellular matrix, including proteoglycans, fibronectin and laminin. Increased expression of MMPs occurs during physiological remodelling of connective tissue, such as in embryogenesis, uterine involution *post partum*, and wound healing, and in disease processes such as arthritis, tumour invasion and periodontitis [3,4].

Inflammatory mediators such as interleukin-1 (IL-1) [5], tumour necrosis factor- α (TNF- α) [6] and leukoregulin [7] induce collagenase- and stromelysin-gene expression in a variety of cells. Furthermore, cellular stress induced by phagocytosis of monosodium urate crystals, treatment with phorbol esters, or heat shock, also stimulate collagenase- and stromelysin-gene expression in cultured fibroblasts [8–10]. Elevated temperature stimulates the rapid synthesis of heat-shock proteins (HSPs) in many cell types. These proteins may play a role in protecting cells from physical and chemical stresses [11]. In contrast with its stimulatory effects on HSP synthesis, heat shock suppresses the transcription of previously active non-heat-shock genes, and the translation of pre-existing mRNAs. Here, we investigated the effects of heat shock on collagenase-gene expression in cultured human dermal and synovial fibroblasts and in NIH/3T3 cells. Our results showed that exposure of cells to heat resulted in a delayed increase in collagenase mRNA during the recovery from

heat shock. This effect was mediated, in part, at the level of transcription and involved an AP-1 binding site in the collagenase promoter.

MATERIALS AND METHODS

Cell cultures

Dermal fibroblast cultures were established from skin biopsies obtained from the dorsal forearm of healthy adults by standard tissue-culture techniques, as described previously [12]. Cells were grown in Eagle's minimal essential medium supplemented with 10 % fetal-bovine serum (Gibco, Grand Island, NY, U.S.A.), 1 % vitamins and 2 mM L-glutamine. Synovial fibroblast cultures were established from normal synovial tissue obtained from patients undergoing leg amputations. Cells were used for experiments between passages 6 and 11. In some experiments, NIH/3T3 mouse cells purchased from the American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.) were used. Synovial fibroblasts and NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium with 10 % fetal-bovine serum, 1 % vitamins and 2 mM L-glutamine. All cells were maintained at 37 °C in a moist atmosphere of air/CO₂ (19:1), and the medium was changed twice weekly. For experiments, cells were grown in 25 cm² or 75 cm² culture flasks until confluency. The caps were closed tightly to minimize loss of CO₂; the flasks were sealed in plastic bags and then submersed in a thermostat-controlled water bath maintained at a constant temperature of either 42 ± 0.5 °C or 44 ± 0.5 °C for the indicated times. As has been shown previously [13], in contrast with human cells, NIH/3T3 cells did not tolerate 44 °C and were exposed only to 42 °C. After heat shock, the flasks were returned to the incubator at 37 °C, and cultures were harvested at different time points. Some cultures were pretreated

Abbreviations used: MMP, matrix metalloproteinase(s); IL-1, interleukin-1; TNF- α , tumour necrosis factor- α ; PMA, phorbol 12-myristate 13-acetate ('TPA'); TRE, TPA-responsive element; HSP, heat-shock protein(s); CAT, chloramphenicol acetyltransferase; TGF- β , transforming growth factor- β .

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Figure 1 Schematic representation of the collagenase CAT constructs employed for transient transfection experiments

Key: 3.8 c'gse CAT = 3.8 kb of the 5' region of the human collagenase gene (−3800 to +37 bp) in pUCAT vector [19]; 475 bp c'gse CAT = 475 bp of the human collagenase gene from −517 to −42 bp relative to the transcription start site ligated to the HSV tk promoter from position −109 to +51 bp and SV40 polyadenylation signal in pBL CAT2 vector [16]; TRE CAT = an 18 bp oligonucleotide 5'-AGCTTGATGAGTCAGCCG-3' containing the TRE sequence (underlined) ligated to the HSV tk promoter from position −109 to +51 bp and SV40 polyadenylation signal in pBL CAT2 vector [20]; ΔTRE CAT = identical with TRE CAT, except for a T→G mutation (at −72 bp), which interferes with AP-1 binding [20].

with cycloheximide (10 µg/ml) (Sigma Chemical Co., St. Louis, MO, U.S.A.) 3 h before exposure to heat. Cellular viability at the end of the incubation period was determined by Trypan Blue dye exclusion.

Extraction of RNA and Northern-blot analysis

Total RNA was isolated by acid guanidinium isothiocyanate/phenol/chloroform extraction [14]. The amount of RNA was quantified by spectrophotometric absorbance at 260/280 nm. Samples containing equal amounts of RNA (10–15 µg) were electrophoresed in 0.8% agarose gels under denaturing conditions. Equal loading of RNA was evaluated by staining with ethidium bromide, to reveal the 18 S and 28 S rRNA subunits under UV light. After electrophoresis the RNA was transferred in a vacuum-blotting system (LKB 2016 Vacugene; Bromma, Sweden) to nitrocellulose filters (Nitroplus MSI; Westboro, MA, U.S.A.). RNA bound to nitrocellulose was UV-crosslinked (UV Stratalinker 2400; Stratagene, La Jolla, CA, U.S.A.). Equal transfer of RNA was assessed visually by UV illumination of rRNA bands after transfer to the filters. Northern hybridizations were performed with cDNAs radiolabelled with [α - 32 P]dCTP by nick translation to a specific radioactivity of $> 10^8$ c.p.m./µg of DNA [15]. Prehybridizations and hybridizations were performed in a solution containing 50% formamide, 5×SSPE [SSPE = 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 5×Denhardt's solution (1×Denhardt's solution contains 0.1% polyvinylpyrrolidone, 0.1% BSA and 0.1% Ficoll), 0.5% SDS, and 250 µg/ml denatured salmon sperm DNA. Hybridizations were performed at 42 °C for 18–24 h. The filters were washed at 68 °C with a final stringency of 0.1×SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS, and were exposed to X-ray films (Eastman Kodak Co., Rochester, NY, U.S.A.) in cassettes with intensifying screens (Kodak) at −70 °C for 18–24 h. The autoradiograms were scanned by densitometry at 633 nm with a laser scanner (LKB). The fol-

owing human cDNAs, all obtained from A.T.C.C., were used: collagenase [16], cytoplasmic 7 S RNA [17] and HSP-70 [18].

Transient transfections and chloramphenicol acetyltransferase (CAT) assays

For transient transfection experiments, the 3.8 kb c'gse CAT [19], 475 bp c'gse CAT [16], TRE [TPA (PMA; phorbol 12-myristate 13-acetate)-responsive element] CAT and ΔTRE CAT [20] collagenase-CAT constructs were used (Figure 1). As controls, pSV2 CAT (a construct containing the SV40 promoter and enhancer-ligated to the CAT gene), the promoterless construct pSV0 CAT, and pBL CAT2 (a construct containing the HSV-tk promoter ligated to the CAT gene) were used.

Human synovial fibroblasts and NIH/3T3 cells were grown in 25 cm² flasks to 80% and 50% confluency, respectively. Cells were then transfected with 10–15 µg of plasmid DNA by the calcium phosphate/DNA co-precipitation method, followed 3 h later by glycerol (15%) shock for 60 s, as described previously [21]. To determine the transfection efficiency, construct pSV2 AP containing the SV40 promoter and enhancer-ligated to a rat alkaline phosphatase cDNA (provided by Dr. K. Yoon) was co-transfected in all experiments [22]. In some experiments, flasks were pretreated with dexamethasone (0.2 µM) (Sigma) 3 h before addition of plasmid DNA. Then 24 h after the transfections, the cells were exposed to heat for 1 h at 44 °C as described above, and then returned to 37 °C for an additional 24 h. The cells were then harvested and the protein concentration in each extract was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Identical amounts of protein (10–20 µg/assay) from each cell extract were used for parallel determination of CAT activity, with [14 C]chloramphenicol as substrate [23]. Acetylated and non-acetylated forms of radiolabelled chloramphenicol were separated by TLC, and revealed by autoradiography. Alkaline phosphatase activity was determined in each extract, as described by Yoon et al. [22]. The proportion of [14 C]chloramphenicol converted into the acetylated forms was determined by cutting out appropriate areas from the TLC sheets and assaying 14 C radioactivity by scintillation counting. The values obtained were expressed as percentage acetylation. The CAT activity in each sample was normalized to alkaline phosphatase activity to correct for small differences in transfection efficiency.

Electrophoretic-mobility-shift analyses

Synovial fibroblasts grown to confluency in 175 cm² flasks were exposed to heat at 42 °C for 3 h, and returned to 37 °C. Nuclear extracts were prepared from cells harvested 3 h or 12 h after heat shock, exactly as described previously [24]. In a typical experiment, a confluent 175 cm² flask (10⁸ cells) yielded ~100 µg of crude nuclear protein. Protein concentrations were determined by a dye-binding assay, and the nuclear extracts were stored in small batches at −70 °C until used. Nuclear extracts were incubated with a synthetic 21 bp double-stranded oligonucleotide (5'-CGCTTGATGAGTCAGCCGGAA-3') containing the human c-jun AP-1 binding site (Promega Biotec, Madison, WI, U.S.A.) which had been end-labelled with [γ - 32 P]ATP by using polynucleotide kinase (Boehringer, Mannheim, Germany). Each binding reaction contained 5–10 µg of nuclear extract, 1–2 µg of double-stranded poly[d(I-C)] (Pharmacia/LKB Biotechnology), and radiolabelled probe containing ~50000 c.p.m. (approx. 0.5 ng). Competition studies were performed with 20-, 50- and 100-fold molar excesses of unlabelled AP-1 binding site oligonucleotides, or with consensus Sp1 binding-site oligonucleotides (Promega). The reaction mixtures were incubated for 30 min on

ice in a buffer containing 60 mM KCl, 10 mM Hepes (pH 7.9), 1 mM dithiothreitol, 1 mM EDTA and 5% glycerol in a total volume of 10 μ l. After incubation, the DNA-protein complexes were resolved from the free probes in non-denaturing 5% polyacrylamide gels [25]. Electrophoresis was performed in 1 $\times$ Tris/glycine buffer (50 mM Tris/HCl, 260 mM glycine) at 30 mA for 120 min. The gels were dried under vacuum and exposed to X-ray film with intensifying screens at -70°C for 16–24 h. Autoradiographs were quantified by densitometry.

RESULTS

Effects of heat shock on HSP-70 and collagenase mRNA levels in dermal and synovial fibroblasts

The effects of heat shock on steady-state HSP-70 and collagenase mRNA levels were examined in confluent monolayer cultures of synovial and dermal fibroblasts. In synovial fibroblasts, exposure to 42°C or 44°C for 1 h caused a marked induction of HSP-70 mRNA, which reached a peak at 1 h and returned to control levels within 24 h after heat shock (Figure 2). Identical results (not shown) were noted when dermal fibroblasts were heated. Collagenase mRNA was barely detectable in untreated fibroblasts. Heat shock was followed by an increase in collagenase mRNA levels, which was detectable at 3 h and reached a maximum at 24 h after exposure of synovial fibroblasts to heat (Figure 2). In dermal fibroblasts, an increase in collagenase mRNA levels was detected only 24 h after exposure of cells to heat (results not shown). Pretreatment of both types of fibroblasts with cycloheximide did not prevent the induction of HSP-70 mRNA. In contrast, cycloheximide completely abolished the induction of collagenase mRNA during recovery from heat shock (results not shown). In the presence of cycloheximide, heat-shock-induced HSP-70 mRNA levels remained elevated for at least 24 h. No changes in cell viability or morphology were detected by Trypan Blue exclusion or phase-contrast microscopy after heat shock.

Figure 2 Effects of heat on HSP-70- and collagenase-gene expression in fibroblasts

Confluent cultures of human synovial fibroblasts were exposed to heat (44°C) for 1 h, and then returned to 37°C . Total RNA was extracted at the indicated times after heat shock, and analysed by Northern hybridizations with HSP-70, collagenase (C'GSE) and cytoplasmic 7 S RNA cDNAs as described in the Materials and methods section. Lanes 1, control (37°C); 2, heat shock, 1 h recovery; 3, heat shock, 3 h recovery; 4, heat shock, 12 h recovery; 5, heat shock, 24 h recovery.

Figure 3 Inducibility of collagenase promoter activity in fibroblasts following heat shock

Human synovial fibroblasts were transiently transfected with the collagenase CAT constructs shown in Figure 1. At 24 h after heat shock (1 h at 44°C), CAT activity was determined as described in the Materials and methods section. Co-transfections with the pSV2 AP construct described in the Materials and methods section were performed to correct for transfection efficiency. The Figure shows an autoradiograph of a representative experiment. The percentage of acetylation, determined from quantification of the acetylated (Ac) and non-acetylated (C) forms of [^{14}C]chloramphenicol by TLC, corrected for alkaline phosphatase activity in each sample, is shown at the bottom of the Figure.

Effect of heat shock on collagenase promoter activity

To study the mechanisms involved in stimulation of collagenase gene expression during recovery from heat shock, transient transfections were performed. The construct 3.8 kb c'gse CAT employed in these experiments contains 3.8 kb of the 5'-flanking DNA of the human collagenase gene, encompassing the AP-1 binding site (TPA-response element, TRE), as well as further upstream putative transcriptional regulatory motifs. Heat shock (44°C for 1 h) of transfected synovial fibroblasts was consistently followed by a greater than 2-fold increase in CAT activity compared with untreated cells (Figure 3).

To identify elements within the 5'-flanking sequence of the collagenase gene responsible for transcriptional activation during recovery from heat shock, constructs containing portions of the collagenase promoter ligated to the HSV thymidine kinase (tk) promoter-CAT reporter gene were used. The pBL CAT2 plasmid containing only the tk promoter was employed in parallel transfections, to ensure the specificity of the heat effect on the collagenase gene. Heat shock of synovial fibroblasts transfected with a construct containing a 475 bp segment of the collagenase promoter ($-517/-42$ bp), or a construct containing a consensus AP-1 binding site from the human c-jun gene, was followed by a greater than 2-fold increase in CAT activity (Figure 3, and results not shown). Activation of the TRE construct, as well as of the construct containing 3.8 kb of the collagenase promoter, during recovery from heat shock, was prevented by pretreatment of cells with dexamethasone (results not shown).

To examine further the role of the AP-1 binding site in heat-induced collagenase-gene expression, we studied the effect of heat shock on the CAT activity driven by a TRE sequence containing a T $\rightarrow$ G mutation (Δ TRE) designed to interfere with AP-1 binding. The transcriptional activity of the TRE CAT was increased ~ 2.5 -fold after heat shock in transiently transfected human synovial fibroblasts, whereas the mutation in the TRE

Figure 4 Analysis of transcription activity of wild-type and mutated TRE CAT constructs following heat shock

Human synovial fibroblasts were transiently transfected with the TRE CAT and Δ TRE CAT constructs. At 24 h after transfection, the cells were exposed to heat for 1 h at 42 °C. After further incubation for 24 h at 37 °C, cells were harvested, and CAT activity of the cell extracts was determined as described in the Materials and methods section. A representative autoradiogram is shown. The percentage of acetylation was determined from quantification of the acetylated (Ac) and non-acetylated (C) forms of [14 C]chloramphenicol, and is shown at the bottom of the Figure.

Figure 5 Effect of recovery from heat shock on nuclear protein binding to AP-1 binding site

Human synovial fibroblasts were exposed to heat (3 h at 42 °C) or left untreated (37 °C). At 3 or 12 h after heat shock, nuclear extracts were prepared, and 5 μ g of nuclear extract was incubated with a radiolabelled 21 bp oligonucleotide containing the human c-jun AP-1 binding site. DNA-protein complexes were analysed by electrophoresis in non-denaturing polyacrylamide gels and autoradiography. A representative autoradiogram is shown. The band at the bottom of the gel represents free oligonucleotide (F); B refers to DNA-protein complexes. The recovery times of cells after heat treatment and the concentrations ($\times$ = fold) of unlabelled competitors AP-1 and SP1 are indicated (C, untreated control). Where indicated, an excess of unlabelled AP-1 binding-site probe was used in the reaction mixture.

sequence completely abolished induction of transcriptional activity (Figure 4). Identical results (not shown) were obtained in transient transfection experiments with NIH/3T3 cells.

Effect of heat shock on nuclear protein binding to the AP-1 site

The results from transfection experiments demonstrated that the AP-1 binding site was able to drive increased CAT activity in fibroblasts after heat shock. To study further the role of the AP-1 site in this response, electrophoretic-mobility-shift assays were performed. Nuclear extracts were prepared from heat-shock-treated synovial fibroblasts that had been allowed to recover at 37 °C. After incubation of nuclear extracts with radiolabelled probes corresponding to the human c-jun AP-1 binding site, DNA-protein complexes were resolved by electrophoresis. The results indicated that heat shock was associated with the delayed induction of nuclear DNA-binding activity specific for the AP-1 binding site (Figure 5). The DNA-protein complex formed in heat-shock-treated cells was increasingly competed away with molar excesses of the unlabelled AP-1 binding-site oligonucleotides, but not with excess of Sp1 binding-site oligonucleotides (Figure 5).

DISCUSSION

Here, we investigated the effects of heat on collagenase-gene expression in human dermal and synovial fibroblasts. Elevated temperature is a form of physical stress that influences physiological processes in various cells. In the present study, we confirmed that heat shock of fibroblasts caused a rapid and transient increase in HSP-70 mRNA levels. Although cycloheximide did not abrogate the induction of HSP-70 mRNA by heat, consistent with a previous report [25], it prevented the rapid decline in HSP-70 mRNA levels. After heat shock, a delayed increase in collagenase mRNA levels was observed. The induction of collagenase mRNA was observed only during an extended recovery, and was completely abolished by pretreatment of the fibroblasts with cycloheximide. Therefore protein synthesis *de novo* appears to be required for heat induction of collagenase-gene expression, but not of HSP-70 gene expression.

By using human collagenase-CAT constructs in transient transfections, a greater than 2-fold increase in promoter activity was observed during recovery from heat shock. The magnitude of the increase in CAT activity after heat shock was considerably lower than the increase in collagenase mRNA levels in heat-shocked compared with untreated fibroblasts. These observations indicate that, in addition to transcriptional activation, other mechanisms, such as alterations in mRNA stability, may also be operative in these fibroblasts. A short DNA sequence corresponding to the AP-1 binding site was sufficient to drive increased CAT activity after heat shock, whereas the Δ TRE CAT construct, containing a T→G substitution within the AP-1 binding site, was unable to respond to heat shock. These data indicate that the AP-1 binding site is involved in the up-regulation of collagenase-gene expression after heat shock. The role of the AP-1 binding site in this effect was supported by results of electrophoretic-mobility-shift assays, indicating that heat shock was associated with increased nuclear DNA-binding activity specific for the AP-1 binding site.

Our results demonstrate that activation of collagenase gene expression during recovery of human dermal and synovial fibroblasts from heat shock is mediated in part at the level of transcription, and involves the AP-1 binding site. This sequence is a binding site for a dimeric DNA-binding protein composed of members of the Jun and Fos families of oncoproteins, and has been implicated in the regulation of the collagenase gene expression by IL-1, TNF- α , phorbol esters, leukoregulin and glucocorticoids [26,27]. Heat shock has been shown to induce c-fos mRNA expression in 3T3 cells [28]. In light of these observations, our present results suggest that induction of

collagenase-gene expression during recovery from heat shock may involve increased c-fos protein synthesis, leading to AP-1 binding to the collagenase promoter.

The expression of a number of genes has been shown to be activated by heat shock in eukaryotic cells. In addition to HSPs, heat shock induces mRNAs for cellular enzymes such as haem oxygenase [29], tyrosine phosphatase [30] and spermidine/spermine N¹-acetyltransferase [31], as well as for c-fos proto-oncogene [28] and transforming growth factor- β s (TGF β) [32]. Transcriptional up-regulation of most heat-shock-responsive genes characteristically occurs within a few minutes after heat shock [33]. In the present studies, HSP-70 mRNA levels displayed a 'classic' heat-shock response, increasing dramatically within 1 h after a transient heat shock, and declining rapidly after removal of heat stress. In contrast, changes in collagenase mRNA levels followed a different temporal pattern, since the increase was noted only during extended recovery (3 h for synovial and 24 h for dermal fibroblasts) after heat shock. A similar pattern of heat-shock response has been recently described for the delayed expression of TGF β mRNA in cultured cardiac myocytes [32]. We speculate that this non-classic temporal pattern of gene expression reflects the indirect nature of heat-shock-induced stimulation of collagenase or TGF β -gene activity. The apparent requirement for protein synthesis *de novo* in the induction of collagenase-gene expression by heat shock is consistent with this notion. Heat stress in fibroblasts therefore may cause the rapid induction of one set of genes, including HSP-70 and c-fos, whose protein products alone or in combination with other cellular gene products may then cause delayed induction of a set of 'secondary response' genes, such as the collagenase gene. In addition, induction of collagenase-gene expression may depend on the synthesis of proteins, suppressed during heat shock and available only during the recovery, that may then influence the stability of collagenase mRNA.

During inflammation, a cascade of events set in motion by elevation of tissue temperature and the effects of pro-inflammatory cytokines may result in induction of collagenase-gene expression. Furthermore, increased temperature has been shown to activate the enzymic activity of collagenase [34]. Induction of collagenase-gene expression after heat shock may therefore play a role in the degradation of the extracellular matrix in inflammatory diseases.

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