HSF1/*hsp72* pathway as an endogenous anti-inflammatory system

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Abstract We investigated the occurrence and the role of HSF1 activation and inducible *hsp72* expression in the carrageenin pleurisy in the rat. Molecular analysis performed on pleural cells collected from rat pleural cavity after carrageenin challenge revealed increased HSF1 activation and *hsp72* expression. Moreover, local injection of a double-stranded oligodeoxynucleotide (ODN) containing the heat shock element sequence, acting as transcription factor decoy, exacerbated the inflammatory reaction. The exacerbation, induced by wild-type, but not by mutant ODN decoy, was associated to both inhibition of HSF1/DNA binding activity and reduction of *hsp72* expression. In conclusion, this study shows that HSF1 activation and *hsp72* expression both actually occur in acute inflammation and that the remission of the inflammatory reaction is tightly associated to the HSF1-dependent *hsp72* expression, suggesting a a relevant role for the HSF1/*hsp72* pathway as an endogenous anti-inflammatory system.

Key words: Acute inflammation; Carrageenin pleurisy; Heat shock factor 1; Stress protein; Transcription factor decoy

1. Introduction

The overall biological significance of inflammation is that of a defence response. However, the inflammatory reaction may be harmful unless kept under control and driven to remission by cytoprotective mechanisms, collectively termed homeostasis. One of the most relevant of these mechanisms is the synthesis of stress-induced or heat shock proteins (*hsp*), of which *hsp70* exhibits a cytoprotective role in a wide variety of human diseases [1,2]. Regulation of *hsp* genes requires the activation and the translocation to the nucleus of a transregulatory protein, the heat shock transcription factor (HSF), which binds to multiple arrays of heat shock elements (HSEs) located in the promoter region of heat shock genes [2]. The recent cloning of HSF genes in higher eukaryotes has revealed a family of HSFs (from HSF1 to HSF4), but is essentially HSF1 that is activated by various kinds of stress. In the cytoplasm, HSF1 is constitutively present in a non-DNA binding state, which is rapidly converted to the DNA binding form upon exposure to heat shock or other environmental stimuli [3]. HSF1 activation requires trimerisation, acquisition of DNA binding activity, translocation to the nucleus and inducible serine phosphorylation, resulting in stress-induced transcription of heat shock proteins [3].

The wealth of information on the defensive role of the *hsp70* family has been so far almost exclusively derived from in vitro systems and there is so far little evidence in vivo of HSF1 activation and/or *hsp72* expression in inflammatory cells or inflamed tissues. Since inflammation is often associated with local increase in temperature it may be conceivable that HSF1 activation and *hsp72* expression may both occur during an inflammatory reaction.

Recent reports have shown that synthetic double-stranded phosphorothioate oligodeoxynucleotides (ODNs) may act as decoy *cis* elements blocking the binding of transcription factors to promoter regions of their targeted genes and resulting in the inhibition of gene transactivation both in vitro [4,5] and in vivo [6–8]. Thus, we used the decoy strategy as a tool for investigating the role of HSF1 in inflammation.

In the present study we show that HSF1 activation actually occurs in rat carrageenin-induced pleurisy, a model of acute inflammation characterised by exudate formation and cell migration. Furthermore, our results provide evidence, for the first time to our knowledge, that the remission of the inflammatory reaction is closely associated to the HSF1-dependent expression of *hsp72*, since administration of double-stranded ODN decoy to HSF1 inhibits HSF1 activation, reduces *hsp72* expression and exacerbates the inflammatory reaction.

2. Materials and methods

2.1. Animals

Male Wistar rats (Harlan, Italy) weighing 175–200 g were used in all experiments. Animals were provided with food and water ad libitum. The light cycle was automatically controlled (on 07 h, off 19 h) and the room temperature thermostatically regulated to 22 ± 1°C. Prior to the experiments animals were housed in these conditions for 3–4 days to become acclimatised. Animal care was in accordance with Italian and European regulations on the protection of animals used for experimental and other scientific purposes.

2.2. Induction of pleurisy

Animals were slightly anaesthetised with isofluorane (Abbott) and 0.2 ml sterile saline solution or 1% λ-carrageenin (Sigma) suspended in sterile saline were injected into the right pleural cavity. Animals were sacrificed 2, 6, 12, 24 and 48 h after the induction of pleurisy in an atmosphere of CO_2. Pleural exudate from each animal was harvested by washing the pleural cavity with 2 ml of sterile saline containing 5 U/ml heparin (Sigma). Any exudate with blood contamination was rejected. The exudate volumes were measured and results were expressed by subtracting the volume (2 ml of sterile saline) injected in the pleural cavity from total volume recovered. Each exudate sample was then centrifuged at 800×*g* for 10 min and cell pellet

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Abbreviations: *hsp*, heat shock protein; HSF, heat shock factor; HSE, heat shock element; ODN, oligodeoxynucleotide; wt-ODN, wild-type ODN; mut-ODN, mutant ODN; EMSA, electrophoretic mobility-shift assay; COX, cyclooxygenase

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resuspended in saline. Total cell count in each sample was estimated after trypan blue staining using a Burker counting chamber. In some experiments differential cell count was determined in smears by May–Grunwald staining. In some experiments, groups of animals, 24 h after carrageenin injection, received a single pleural injection of 3 nmol of wild-type ODN (wt-ODN) or mutant ODN (mut-ODN) in 0.1 ml of sterile saline, while control rats received saline alone. Then, 24 h later, rats were sacrificed and pleural exudates collected and processed as described above.

2.3. Transcription factors decoy oligonucleotides

The ODNs used in this study were synthesised as phosphorothioate derivatives, which are resistant to degradation by endonucleases, to our specifications by Tib Molbiol Roche Biochemical. Double-stranded phosphorothioate ODN decoy to HSF1 was prepared by annealing of sense and antisense oligonucleotides in vitro in 1× annealing buffer. The mixture was heated at 100°C for 12 min and allowed to cool to room temperature slowly over 18 h.

The phosphorothioate ODN sequences used in this study were: 5′-CTA GAA GCT TCT AGA AGC TTC TAG-3′ for wild-type HSF ODN decoy and 5′-ATA TCC GCA GGT ATC GGC AGT TAG-3′ for mutant HSF ODN decoy.

2.4. Preparation of cell extracts

All the extraction procedures were performed on ice with ice-cold reagents. Cells were separated from the inflammatory exudate by centrifugation at 800×*g* for 5 min at 4°C. Inflammatory cell pellets were washed twice in phosphate-buffered saline (PBS; ICN Biomedicals) and centrifuged at 800×*g* for 5 min at 4°C. Whole cell extracts were prepared by resuspending the pellet in one packed cell volume of a high-salt extraction buffer and incubated on ice for 15 min. After centrifugation at 13 000×*g* at 4°C for 5 min, the protein concentration in the supernatant was determined by the Bio-Rad protein assay kit and then it was aliquoted and stored at −80°C.

Nuclear extracts were prepared as previously described [9]. Protein concentration was determined by the Bio-Rad protein assay kit.

2.5. Electrophoretic mobility-shift assay (EMSA)

A double-stranded oligonucleotide containing the idealised HSE sequence [10–12] was end-labeled with [γ-^{32}P]ATP (ICN Biomedicals) and gel mobility assay was performed as described [12]. Aliquots of nuclear extracts (15 μg protein for each sample) were incubated for 30 min with radiolabeled oligonucleotides (2.5–5.0×10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC (Roche Biochemicals), 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 10% v/v glycerol. The specificity of the DNA/protein binding was determined by competition reaction in which a 50-fold molar excess of unlabeled wild-type, mutant or Oct-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to HSF1 (StressGen) or HSF2 (LabVision) proteins were added to the reaction mixture 30 min before the addition of radiolabeled HSF1 probe. Recombinant human HSF1 protein (StressGen) was used as positive control. Protein–nucleic acid complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at −80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

2.6. Immunoprecipitation and Western blot analysis

The level of *hsp72* was quantified in whole cell extract, by immunoprecipitation followed by Western blot analysis according to the manufacturer's instructions (Santa Cruz). Briefly, equivalent amounts of whole cell extracts (100 μg for each sample) were mixed with 40 μl of protein A-Sepharose and 2 μl of anti-*hsp72* polyclonal antibody (StressGen) which specifically recognises the inducible but not the constitutive members of the *hsp70* family and left overnight at 4°C with continuous shaking. Immunocomplexes were washed three times with 500 μl of buffer A (10 mM Tris–HCl pH 7.5, 1 M NaCl, 0.2% Triton X-100 and 2 mM EDTA), mixed with 40 μl of gel loading buffer (50 mM Tris/10% sodium dodecyl sulphate/10% glycerol/10% 2-mercaptoethanol/2 mg bromophenol per ml) and then boiled for 3 min. Samples so obtained were electrophoresed in a 10% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4°C overnight with 10% non-fat dry milk in PBS and then incubated with anti-*hsp72* antibody for 1 h at room temperature. The membranes were washed three times with 1% Triton X-100 in PBS and then incubated with anti-rabbit immunoglobulin coupled to peroxidase. The immunocomplexes were visualised by the ECL chemiluminescence method (Amersham). Subsequently, the relative expression of *hsp72* was quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

2.7. Statistics

Data are expressed as mean ± S.E.M. for *n* rats. Statistical analysis was done using an ANOVA followed by a Bonferroni *t*-test. The level of statistically significant difference was defined as $P < 0.05$.

3. Results

3.1. Time course of exudate volume and cellular infiltration

The pleural cavity of normal (untreated) rats contained no exudate and 2.9 ± 0.2×10^6 cells per rat ($n = 5$) predominantly mononuclear (> 95%). Injection of 0.2 ml of 1% λ-carrageenin into the pleural cavity of rats caused an inflammatory response characterised by exudate formation and cell migration (Fig. 1). Both the exudate volume and the total leukocyte number migrated into the pleural cavity increased with time, reached the maximum at 24 h (1.57 ± 0.10 ml/rat and 90.6 ± 6.9×10^{6}cells/rat, $n = 9$), and decreased at 48 h (0.30 ± 0.05 ml/rat and 39.9 ± 3.8×10^6 cells/rat, $n = 10$). Differential cell count of leukocytes migrated into the pleural cavity

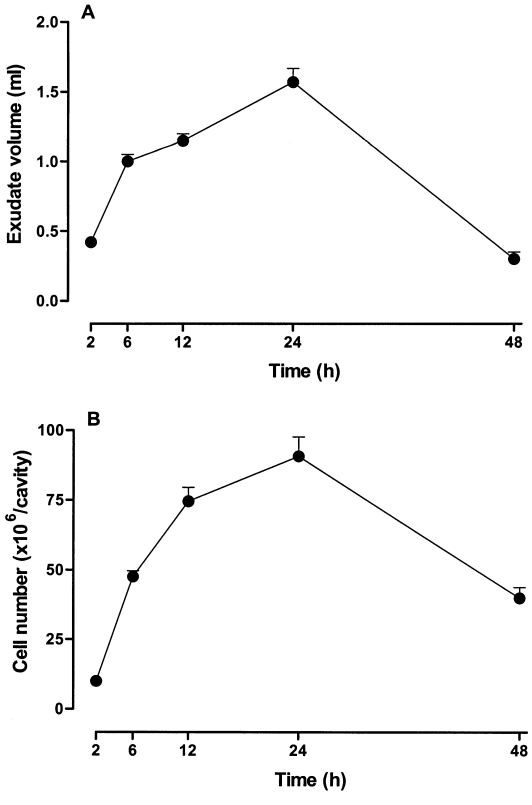

Fig. 1. Time course of rat carrageenin pleurisy. Exudate volume (A) and leukocyte infiltration (B) were evaluated at different time points after carrageenin injection. The values are expressed as mean ± S.E.M. of 7–10 rats.

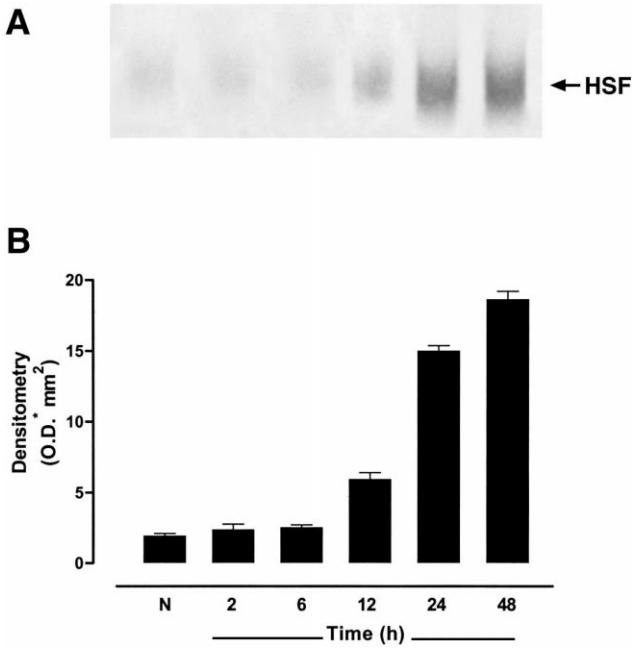

Fig. 2. Time course of HSF/DNA binding activity. EMSA was performed on nuclear extracts from rat pleural cells collected either from normal rats (N) or from carrageenin-treated rats at different time points (2, 6, 12, 24 and 48 h) after pleural injection. EMSA in A is from a single experiment and is representative of three separate experiments. Results of densitometric analysis in B are expressed as mean ± S.E.M. of three separate experiments.

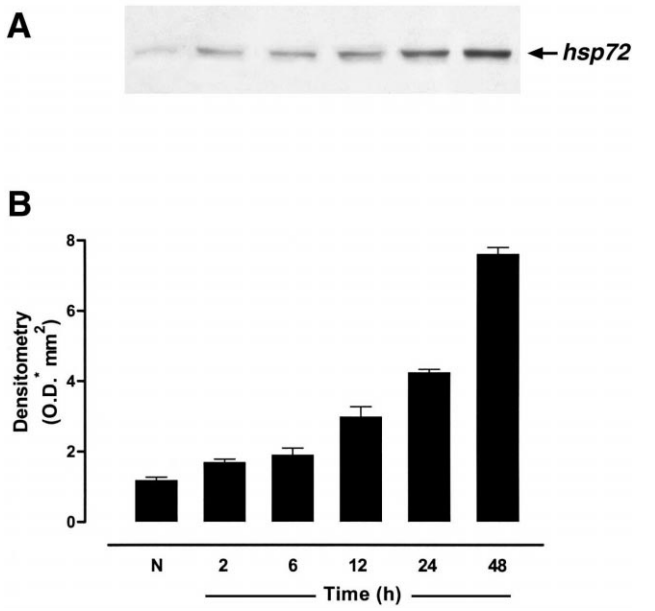

Fig. 3. Time course of *hsp72* expression. Immunoblotting analysis was performed in whole extracts from rat pleural cells collected either from normal rats (N) or from carrageenin-treated rats at different time points (2, 6, 12, 24 and 48 h) after pleural injection. Western blot in A is from a single experiment and is representative of three separate experiments. Results of densitometric analysis in B are expressed as mean ± S.E.M. of three separate experiments.

showed that polymorphonuclear leukocytes dominated the early phase (2 h) of the reaction (91% of polymorphonuclear leukocytes and 19% of mononuclear leukocytes) and were replaced at 48 h by mononuclear leukocytes (29% of polymorphonuclear leukocytes and 71% of mononuclear leukocytes).

3.2. Time course of HSF binding activity and hsp72 protein expression

To detect HSF/DNA binding activity, an EMSA was performed by using nuclear extracts from pleural cells collected from normal rats or at different time points (2, 6, 12, 24, 48 h) after carrageenin challenge and a labeled oligonucleotide containing the idealised HSE sequence. As shown in Fig. 2, a retarded band of DNA–protein complex was faintly detected either in normal rats or in the early phase (2, 6 and 12 h) of carrageenin pleurisy, while it was clearly detected at 24 h and at 48 h.

We also investigated the presence of *hsp72* in the whole fractions of pleural cells collected from normal rats or from carrageenin-treated rats at different time points (2, 6, 12, 24 and 48 h) after induction of the pleurisy. Western blot analysis and its relative densitometric analysis showed a time-dependent increase of *hsp72* protein levels in whole extracts of pleural cells from carrageenin-challenged rats as compared to normal rats (Fig. 3).

3.3. Characterisation of HSF complex induced by carrageenin in rat pleural cells

In order to confirm the specificity of the HSF/DNA complex activated by carrageenin we performed experiments of competition and supershift (Fig. 4). The specificity of HSF/DNA binding complex was demonstrated by the complete

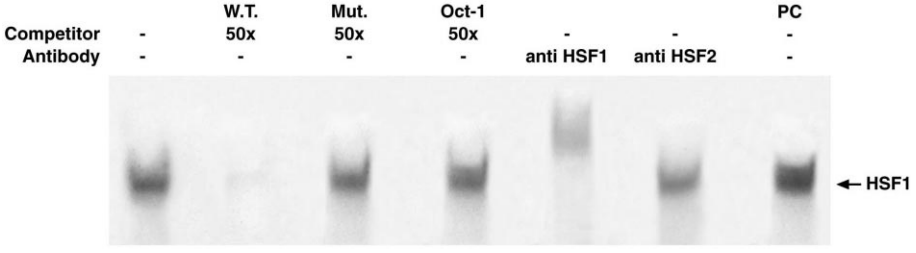

Fig. 4. Identification of carrageenin-induced HSF1/DNA binding activity. Nuclear extracts from carrageenin-elicited inflammatory cells were prepared as described in the text and incubated with ^{32}P-labeled HSF probe. In competition reaction nuclear extracts were incubated with radiolabeled HSF probe in the absence or presence of a 50-fold molar excess of identical but unlabeled HSF probe (W.T. 50×), mutated nonfunctional HSF probe (Mut. 50×) or unlabeled oligonucleotide containing the consensus sequence for Oct-1 (Oct-1 50×). In supershift experiments nuclear extracts were incubated with antibodies against HSF1 or HSF2 30 min before incubation with radiolabeled HSF probe. PC = positive control. Data illustrated are from a single experiment and are representative of three separate experiments.

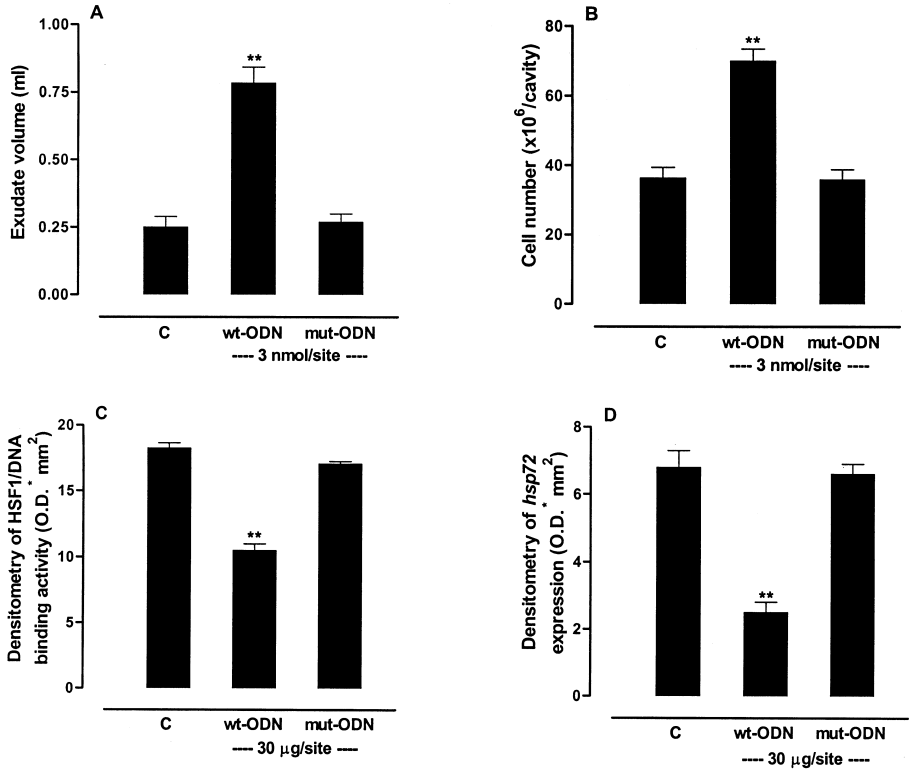

Fig. 5. Effect of ODN decoy on rat carrageenin pleurisy, HSF1/DNA binding activity and *hsp72* protein expression. wt-ODN decoy (3 nmol/site) and mut-ODN decoy (3 nmol/site) were injected into the pleural cavity of rats 24 h after carrageenin injection. Exudate volume (A) and leukocyte migration (B) were evaluated 48 h after carrageenin challenge. Densitometric analysis of HSF1/DNA binding activity measured by EMSA (C) and of *hsp72* protein expression measured by Western blot (D) were evaluated in inflammatory cells collected 48 h after carrageenin challenge. Results in A and B are expressed as mean ± S.E.M. of 6–9 rats. Results in C and D are expressed as mean ± S.E.M. of three separate experiments. $^{**}P < 0.01$ vs control group (C).

displacement of the HSF/DNA binding in the presence of a 50-fold molar excess of unlabeled HSF probe in the competition reaction. In contrast, a 50-fold molar excess of unlabeled mutated HSF probe or Oct-1 oligonucleotide had no effect on this DNA binding activity. Furthermore, to establish which member of the HSF family was activated by carrageenin, nuclear cell extracts were preincubated with specific antibodies for either HSF1 or HSF2 and analysed by EMSA. Addition of anti-HSF1 but not anti-HSF2 to the binding reaction resulted in a supershift of HSF band, thus indicating that carrageenin induces HSF1/DNA binding activity (Fig. 4).

3.4. Effect of ODN decoy on carrageenin-induced rat pleurisy

In preliminary experiments we established that injection into the rat pleural cavity of test agents at doses used in this study did not produce any detectable effect. The average volume of the exudate at 48 h in control animals (carrageenin alone) was 0.25 ± 0.04 ml/rat ($n = 9$) and the total leukocytes number into the pleural cavity was $36.33 \pm 3.12 \times 10^6$cells/rat ($n = 9$). The injection into the pleural cavity of wt-ODN decoy (3 nmol/site), given 24 h after carrageenin challenge, significantly increased both exudate volume to 0.78 ± 0.06 ml/rat ($P < 0.01$; $n = 9$) and total leukocytes number to $70 \pm 3.5 \times 10^6$cells/rat ($P < 0.01$; $n = 9$). In contrast, mut-ODN decoy ($n = 6$) given at the same dose and time as the wild-type had no effect (Fig. 5A,B). Furthermore, neither wt-ODN nor mut-ODN decoy affected the profile of cell population in the exudate fluid which resulted virtually superimposable to that found in control rats, i.e. animal receiving carrageenin alone (see Section 3.1). Thus, 48 h after carrageenin injection the pleural exudate from rats treated with wt-ODN (3 nmol/site, $n = 6$) contained 25% of polymorphonuclear leukocytes and 75% of mononuclear leukocytes, while the exudate from rats treated with mut-ODN (3 nmol/site, $n = 6$) contained 28% of polymorphonuclear leukocytes and 72% of mononuclear leukocytes.

3.5. Effect of ODN decoy on HSF1 activation and hsp72 expression in pleural cells

To detect HSF1/DNA binding activity nuclear extracts from pleural cells collected 48 h after carrageenin injection were analysed by EMSA. Treatment of rats with wt-ODN (3 nmol/site; $n = 9$) caused a significant ($P < 0.01$) inhibition of carrageenin-induced HSF1/DNA binding activity by 42% (Fig. 5C). In contrast, mut-ODN (3 nmol/site; $n = 6$) did not modify carrageenin-induced HSF1/DNA binding activity as compared to control animals (Fig. 5C).

The levels of *hsp72* from pleural cells collected 48 h after carrageenin injection were examined by immunoblotting analysis. Injection of wt-ODN (3 nmol/site; $n = 9$) significantly ($P < 0.01$) reduced *hsp72* band intensity by 63% (Fig. 5D). In contrast, mut-ODN (3 nmol/site; $n = 6$) had no effect on carrageenin-induced increase of *hsp72* levels as compared to control group (Fig. 5D).

4. Discussion

In this study we demonstrate that HSF1-dependent *hsp72* expression occurs in rat carrageenin pleurisy, a model of acute inflammation. Both HSF1 and *hsp72* appear in inflammatory cells collected from the pleural exudate at the late phase of the reaction and are closely associated to and likely control the remission of the process. Moreover, in the present study we report a novel experimental approach to the study of the inflammatory response, i.e. in vivo administration of a decoy *cis* element to bind the transcription factor HSF1. Our results show that injection into the rat pleural cavity of double-stranded ODN as decoy, by competitively inhibiting the binding of HSF1 to native DNA sequence, reduced *hsp72* expression and exacerbated carrageenin-induced pleurisy, suggesting a relevant role of the HSF1/*hsp72* pathway as an endogenous anti-inflammatory system. The specificity of the effect of ODN decoy to HSF1 is supported by evidence showing that ODN decoy with a mutated HSF1 consensus sequence does not affect carrageenin-induced pleurisy.

Heat shock proteins, particularly *hsp70*, act as molecular chaperones, have well established functions in protecting cells, and are responsible for thermotolerance and acquired resistance to other forms of injury. Thus, recently it has been shown that pretreatment of rats with a mild heat shock improved recovery from ischemia of perfused rat hearts [13]. Similarly, in rats submitted to heat shock 24 h earlier, a decrease in myocardial infarct size occurred in comparison to control animals [14].

Since in our experimental protocol rats did not receive any kind of heat shock, the activation of the HSF1/*hsp72* system has been caused only by the stress due to the inflammatory stimulus.

A question that rises from this study is how can inflammation induce the activation of the HSF1/*hsp72* system. It has been speculated that heat shock proteins themselves may regulate heat shock gene expression via an autoregulatory loop [15]. According to this hypothesis, misfolded proteins occurring in large amounts during heat shock or other forms of stress might sequester *hsp70* and lead to activation of HSF1. Support for this autoregulatory hypothesis comes from the observation that inactive HSF1 in cytoplasmic extracts from non-heat-shocked HeLa cells can be converted to the DNA binding state by exposure to environmental stress such as heat, non-ionic detergents or low pH [16,17], whereas the addition of *hsp70* blocks this conversion [18].

However, it has recently been shown, in rheumatoid arthritis synovial tissue [19], that pro-inflammatory cytokines, such as tumour necrosis factor-α, interleukin (IL)-6 and IL-1, which are highly expressed in rat carrageenin-induced pleurisy [20], induce HSF1 activation and *hsp72* expression. Moreover, it has been demonstrated that cyclooxygenase (COX) cyclopentenone metabolites such as prostaglandins of the A and J type induce the synthesis of *hsp70* in a wide variety of human and mammalian cells [21]. Furthermore, it has also been shown that cyclopentenone prostaglandins are generated by COX-2 during the late phase of carrageenin pleurisy in rats and contribute to the resolution of the inflammatory reaction through not yet identified molecular mechanisms [22]. In this light it is conceivable to hypothesise that the activation of HSF1, occurring during the late phase of carrageenin pleurisy, may be induced by cyclopentenone prostaglandins.

Although the anti-inflammatory mechanism of *hsp72* has not been clarified yet we may hypothesise that in our system this protein may drive the inflammatory reaction to the resolution by protecting mononuclear leukocytes which are known to produce endogenous anti-inflammatory molecules such as TGF-β, IL-10 and cyclopentenone prostaglandins [22–24]. Further studies will clarify this hypothesis.

Another important issue addressed by our study is the demonstration that ODN decoy strategy can be used in vivo for investigating the inflammatory reaction. Thus, our results demonstrated that the inhibition of *hsp* genes expression results in exacerbation of the inflammatory process. Therefore these genes may be considered as anti-inflammatory or 'therapeutic' genes, and their selective in vivo transactivation, by appropriate pharmacological agents, may lead to the remission of the inflammatory reaction. In conclusion, our results suggest that the HSF1/*hsp72* pathway may represent an endogenous anti-inflammatory system whose activation protects the organism from the deleterious effects of a prolonged and excessive activation of the inflammatory response. Moreover, the transcription factor decoy strategy, by interfering with the activation and expression of selected genes, may represent a useful tool for a better comprehension of the inflammatory process as well as for future therapeutic approaches.

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