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OSTEOARTHRITIS and CARTILAGE

Analysis of heat shock proteins and cytokines expressed during early stages of osteoarthritis in a mouse model

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Summary

Objective: Osteoarthritis (OA) is a debilitating disease of the joints. The joints of affected individuals are characterized by a progressive degeneration of articular cartilage leading to inflammation and pain. The expression of heat shock proteins (HSPs) is a ubiquitous self-protective mechanism of all cells under stress, furthermore, the synovium of osteoarthritic individuals contains high levels of cytokines. This study seeks to establish the role of HSPs and cytokines in OA.

Methods: We have investigated the presence of HSPs and cytokines in articular cartilage during early stages of OA in a mouse that is known to develop spontaneous OA lesions (C57 black mouse). The articular cartilage from closely related mice (C57BL/6) was used as control. Messenger RNAs (mRNAs) for HSPs (HSP32, HSP47, HSP60, HSP70, HSP84 and HSP86) and cytokines [interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)] were detected by reverse transcription-polymerase chain reaction (RT-PCR).

Results: The mRNA levels of HSP47, HSP70, HSP86, IL-6, and IFN-γ were up-regulated in the cartilage of C57 black mice, whereas, the level of expression of HSP32, HSP60, HSP84 and IL-1β remained unchanged. Furthermore, the expression of IL-1β, IL-6, TNF-α and IFN-γ mRNA was associated with expression of HSP60, HSP47, HSP70 and HSP70/HSP86 mRNA, respectively.

Conclusions: The findings in this study suggest that chondrocytes are conditioned under non-physiological stress during early stages of OA, In addition, among HSPs, HSP70 was associated with two different highly expressed cytokines in C57 black mice, indicating the possible role of HSP70 as a characteristic indicator of early stage of OA.

Key words: Osteoarthritis, Experimental model, Cytokine, Heat shock protein.

Introduction

A prominent event in the development of osteoarthritis (OA) is a progressive degradation of articular cartilage. Molecular analysis has revealed that during OA there is a generalized stimulation of the production of cytokines. The synovium of OA patients contains elevated levels of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [1–3]. Elevated levels of synovial interleukin-6 (IL-6) and TNF-α were also discovered in a canine model of OA [4].

Cells often respond to external stimuli, such as heat, mechanical agitation or chemical toxins, by producing a family of proteins referred to as 'heat shock proteins (HSPs)' or 'stress proteins'. HSPs function to protect the cellular proteins, presumably, by serving as molecular chaperones [5]. HSPs ranging in size from 25–110 kDa have been identified in all animals studied [6]. Families of HSPs are highly conserved in evolution from bacteria to humans [7].

We initiated our studies based on the premise that chondrocytes from OA joints are subjected to stresses that may lead them to initiate their stress response programs by overexpression of HSP and/or cytokine genes. One of us has previously reported that chondrocytes from OA patients overexpress HSP70 (the 70 kDa heat shock protein). Furthermore, the level of HSP70 expression correlated with the severity of OA in humans [8]. There is some evidence that the expression of cytokines correlates with HSP

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expression. For example, expression of IL-1β and HSP70/HSP90 is inversely related *in vitro* [9]. Furthermore, a number of reports have shown that cytokines can stimulate HSP expression [10], since HSP70
expression was induced by IL-1, interleukin-2 (IL-2) and TNF-α [11–13].

We have used an animal model of spontaneous OA in our studies. The C57 black mouse was developed by Silberberg *et al.* in 1941 [14]. OA changes in male C57 black mice (3–24 months) have been investigated in detail [15, 16]. Specifically, OA changes were observed in 60% of knee joints from 6 month old C57 black mice, whereas OA developed to 93% in 12 month old C57 black mice and progressed to 100% in mice aged 18–24 months [17]. All OA changes in 6 month old and in 93% of 12 month old C57 black mice were grade 1 according to modified Wilhelmi's classification [18]. Therefore, we decided to use 6–12 month old C57 black mice as models of early stage of OA. C57 black mice develop OA in the lateral tibial plateau, with greater susceptibility in males than in females, hence the use of males in this study. We have also examined a closely related strain, C57 BL/6 mouse as a control.

In this study, mRNAs for HSPs and cytokines [i.e., HSP32, 47, 60,70, 84, 86, IL-1β, IL-6, TNF-α and interferon-γ (IFN-γ)] were detected in the articular cartilage of C57 black mice and C57 BL/6 using the highly sensitive technique of reverse transcription-polymerase chain reaction (RT-PCR). Moreover, we present data implicating a correlation between HSP expression and cytokine expression during experimental OA.

Materials and Methods

ANIMALS

Male C57 black mice (provided by CIBA-GEIGY Limited, Basel, Switzerland) and C57 BL/6 (obtained from Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) were used. The animals were kept in cages on a wood chip bedding in an air conditioned room at constant temperature with free access to food and water. This experimental procedure was permitted by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

HISTOLOGICAL ANALYSES

Ten C57 black mice were euthanized at 6 months, and seven C57 black mice were euthanized at 8–12 months (C57 black mice group). Fourteen C57 BL/6 mice (6 months of age) were also euthanized (BL/6 group). Cartilage tissue of the right knees were fixed in 4% paraformaldehyde/phosphate-buffered solution (PBS) (pH 7.4), decalcified in 10% EDTA/PBS, and embedded in paraffin wax. Sagittal whole knee-joint sections were made (7 μm) on both lateral and medial sides, and stained with Safranin O and fast green or hematoxylin and eosin. The severity of OA in the articular cartilage of the mouse knee joint was classified into five grades (grades 0, 1, 2, 3, and 4) (modified from Wilhelmi's classification) (Fig. 1) [18].

PREPARATION OF RNA AND cDNA

Cartilage tissue from the lateral side of the left proximal tibial plateau was collected. The surrounding soft tissue around the cartilage was detached, and subchondral bone was removed under a stereoscopic microscope. Subsequently, about 10 mg of cartilage tissue from each mouse was individually homogenized by a small mortar and pestle in 0.5 ml of 4 M guanidinium thiocyanate containing 25 mM sodium-citrate, 0.5% sodium-sarkosyl and 0.1 M 2-mercaptoethanol. Then, about 7 μg of total RNA was extracted by the acid guanidinium thiocyanate–phenol chloroform method as previously described [19]. One microgram of total RNA was incubated at 65°C for 5 min, chilled on ice, and reverse-transcribed in a final volume of 10 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 μM each of dATP, dCTP, dGTP, and dTTT (Pharmacia Biotech AB., Uppsala, Sweden), 1 μM oligo $(dT)_{16}$ primer, 20 U RNasin (Ribonuclease inhibitor, Toyobo Co., Ltd., Osaka, Japan), and 100 U MoMuLV RNase H–reverse transcriptase (GIBCO BRL Co., Gaithersburg, MD, USA). The mixture was incubated at 43°C for 1.5 h, heated to 95°C for 10 min, and stored at −20°C.

POLYMERASE CHAIN REACTION (PCR)

The cDNA preparations were analyzed by using a PCR method for mRNA expression of HSP32, 47, 60, 70, 84, 86, IL-1β, IL-6, TNF-α and IFN-γ. In order to ensure the quality of the RNA preparation and to determine the feasibility of the RT-PCR protocol, β-actin RT-PCR products were amplified for all mice. cDNA was heated to 95°C for 10 min and cooled on ice for 5 min. cDNA (5 μl) was added to a 50 μl reaction mixture containing 5 μl

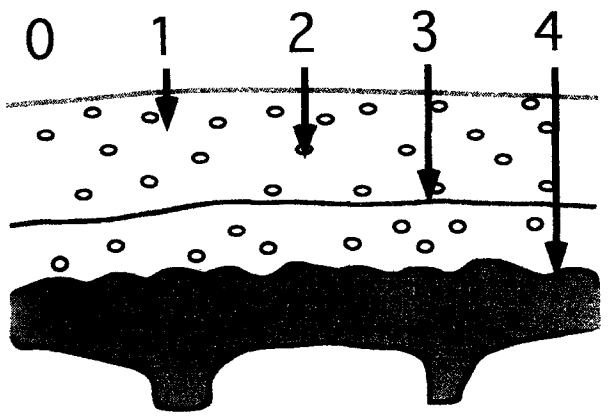

FIG. 1. Classification of severity of OA in the articular cartilage of the mouse knee joint. Grade 0: age related changes. Grade 1: superficial irregularity and erosion of the cartilage. Grade 2: ulceration extending into the deep strata of uncalcified cartilage. Grade 3: ulcers extending into the calcified cartilage. Grade 4: defects of the entire cartilage extending into the bone. (Modified from Wilhelmi, 1978 [18]).

10×PCR reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.8), 15 mM $MgCl_2$, 1% triton X-100, 200 μM of each dATP, dCTP, dGTP and dTTT (Pharmacia Biotech AB.), 200 nM of each priming oligomer, 1.0 U Taq DNA polymerase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and H_2O]. Twenty-five microliters of mineral oil was layered over the aqueous phase to prevent evaporation. Amplification was performed using a DNA thermal cycler (Thermal Cycler, Hybaid Ltd., UK) for 35 cycles. A cycle profile consisted of 1 min at 95°C for denaturation, 1 min at 60°C for annealing, and 1 min at 72°C for extention. Electrophoresis of 10 μl of the reaction mixture on a 1.5% agarose (Dojin Chemical Co., Ltd, Kumamoto, Japan) gel containing ethidium bromide was performed to evaluate amplification and size of generated fragments. A 123 bp DNA ladder (GIBCO BRL Co.) was used as a standard size marker.

Oligonucleotide primer sets for HSP32, 47, 60, 70, 84, 86, IL-1β, IL-6, TNF-α, IFN-γ and β-actin were as described previously (Table I) [20–23].

STATISTICAL EVALUATION

Differences in prevalence of cytokine and HSP expression between groups, and mutual association of cytokine and HSP expression were analyzed statistically. All combinations of 2×2 cross tables were determined using Fisher's exact probability test.

Results

INCIDENCE OF OA CHANGES BY HISTOLOGICAL EXAMINATION

Early stage OA changes were observed in 60% of the tibial plateau of C57 black mice at the age of 6 months, and in 86% of mice at the age of 8–12 months. In contrast, only 36% of BL/6 mice at the age of 6 months show OA changes (Table II). All of OA changes occured in the lateral side, except in two mice aged 8–12 months which showed both lateral and medial lesions. Most of OA changes indicated surface irregularity and loss of chondrocytes, mainly in the superficial layer, which were evaluated as grade 1 by modified Wilhelmi's classification. Two cases of C57 black mice showed cartilage damage extending to the deep layer of uncalcified cartilage, and were evaluated as grade 2 (Fig. 2). The *P*-values were determined on each OA grade (Table II).

DETECTION OF MRNAS FOR HSPS AND CYTOKINES BY PCR

Bands of the amplified PCR products for all cytokines and HSPs were confirmed at the expected sizes in some cases of C57 black mice following agarose gel electrophoresis (Fig. 3). PCR products for β-actin were clearly detected in RNA preparations from all mice in the C57 black mice

Table I.
Cytokine and HSP-specific oligonucleotide primers for PCR

Cytokine	Primer*	Position	cDNA	Ref.†
HSP32	S	609-628	554	20
	AS	1143-1162		
HSP47	S	641-660	607	21
	AS	1227–1247		
HSP60	S	765-784	585	20
	AS	1330-1349		
HSP70	S	2020-2039	648	20
	AS	2648-2667		
HSP84	S	1421-1440	545	20
	AS	1946-1965		
HSP86	S	769-788	919	20
	AS	1668-1689		
Il-1β	S	1895-1916	345	22
	AS	4305-4326		
IL-6	S	1537-1558	211	22
	AS	2979-3000		
TNF-α	S	4536-4557	678	22
	AS	6174-6196		
IFN-γ	S	131-148	401	22
	AS	514-531		
β-actin	S	279-302	762	23
	AS	1017-1040		

*S, sense; AS, antisense.
†Literature from which the sequence data were obtained.

Table II
Incidence and severity of OA changes

OA grade	C57 black mice group 8–12 months $N=7$	C57 black mice group 6 months $N=10$	BL/6 group 6 months $N=14$	P-value
0	1	4	9	$P=0.05$
1	5 (1)	5 (0)	5 (0)	$P>0.17$
2	1 (1)	1 (0)	0	$P>0.30$
3	0	0	0	
4	0	0	0	
1–4	86%	60%	36%	

The severity of OA in the articular cartilage of the mouse tibial plateau was evaluated by modified Wilhelmi's classification. The number in parenthesis showed the number of mice with bilateral OA changes. P-values between C57 black mice group and BL/6 group on each grade are shown in right lane. Significant difference was observed at OA grade 0.

preparations from all mice in the C57 black mice and BL/6 groups, which confirmed exact RNA preparation [Fig. 4(a)].

PREVALENCE OF CYTOKINES AND HSPS mRNA EXPRESSION

The prevalence of mRNA expression of HSPs and cytokines studied here is depicted in Table III. Prevalence refers to the percentage of mice, studied in any given group, that are positive for the expression of the HSPs or cytokines under consideration. Differences in the prevalence of HSP and cytokines mRNA expression were analyzed between the C57 black mice group as a whole (6–12 months) and BL/6 group (6 months), age matched C57 black mice (6 months) and BL/6 groups (6 months), 8–12 month old C57 black mice group and BL/6 group, and between the two age groups of the C57 black mice (6 months and 8–12 months), respectively. Prevalence of HSP47, HSP70, HSP86 and IFN-γ mRNA expression was significantly higher in C57 black mice group as a whole than in the BL/6 group. Among age-matched animals, prevalence of HSP70, HSP86 and IFN-γ was significantly higher in the C57 black mice group (6 months) than in the BL/6 group (6 months). Interestingly, prevalence of HSP47 mRNA expression was significantly higher only in 8–12 month old C57 black mice group when compared with BL/6 group or 6 month old C57 black mice group. TNF-α mRNA was expressed with higher prevalence in C57 black mice group as a whole than in the BL/6 group. Expression of the mRNA of HSP32, HSP60, HSP84 and IL-1β was observed in almost all animals. Significance was assigned when P-values were less than 5%. The results of electrophoresis for HSP47 and 84 mRNA expression are shown in Fig. 4(b) and 4(c), respectively.

There was no significant association between histological OA changes and cytokines or HSPs mRNA expression.

ASSOCIATION OF CYTOKINE mRNA EXPRESSION WITH HSP mRNA EXPRESSION

Association of HSP expression to each cytokine was analyzed statistically (Table IV). HSP70 mRNA expression was associated with TNF-α and IFN-γ mRNA expression. High prevalence of HSP47, 60 and 86 expression was associated with IL-6, IL-1β and IFN-γ, respectively. P-values less than 1% were considered to be significant.

Discussion

The molecular mechanisms related to the progressive cartilage degeneration in OA remain to be elucidated. In this study, we have used the powerful and sensitive technique of RT-PCR to identify the expression of various HSPs and cytokines in OA.

To facilitate a thorough study, we chose the C57 black osteoarthritic mouse as an animal model. Our reasons for choosing this animal model were several fold. Although a wide variety of other animals have been used as models of OA, most require some form of physical intervention for the development of OA lesions, such as ligament section [24], meniscectomy [25], intra-articular injection [26] or immobilization [27]. Therefore, the development of OA in these models might represent a traumatic secondary OA. In comparison, inbred C57 black mice develop OA spontaneously [15–17], leading to consistency of OA

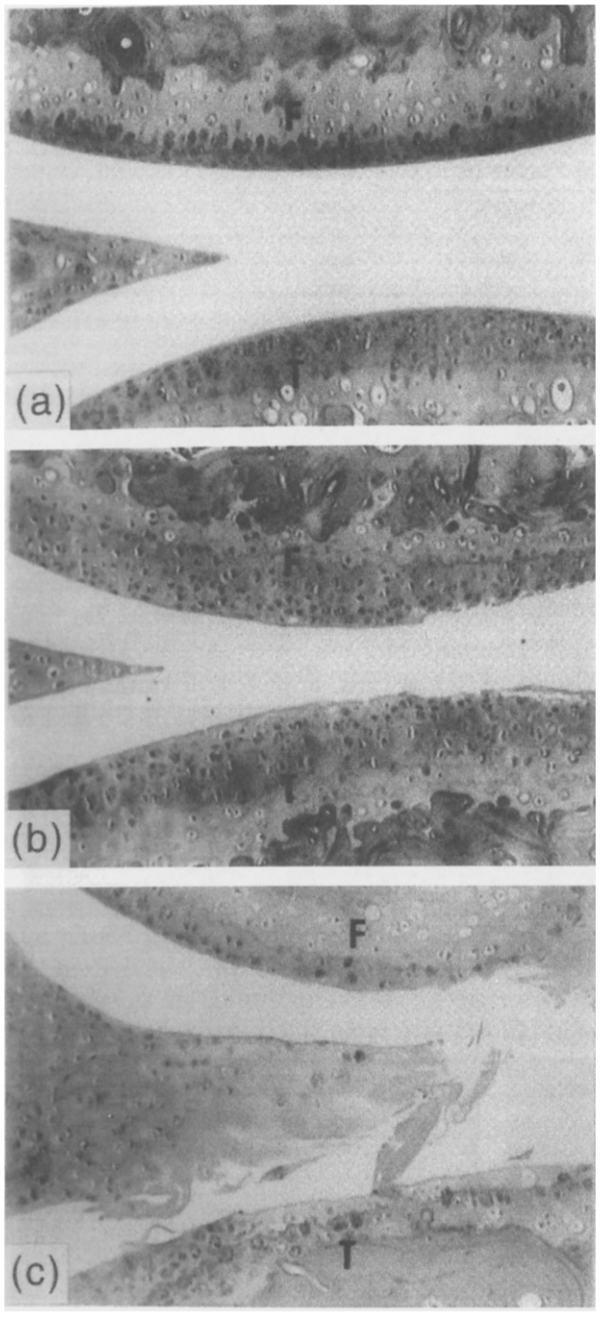

FIG. 2. Sagittal whole knee joint sections stained with hematoxylin and eosin. Severity of OA was evaluated by modified Wilhelmi's classification. (a) The section evaluated as grade 0, which showing smooth cartilage surface and chondrocytes were dense. (b) The section indicated grade 1. Most of the OA changes showed only the lesions with surface irregularity and loss of chondrocytes mainly in the superficial layer. (c) The section showed grade 2. Cartilage damage extends to deep layer of uncalcified cartilage.

development and reproducibility of histological changes. Furthermore, the C57 black mouse model develops OA in the knee joints slowly as compared with other mouse model of OA, such as STR/IN [28] and STR/ORT [29]. The histological examination in this study revealed that C57 black mice had a higher prevalence of OA changes in the tibial plateau at 6 months than C57 BL/6. Most OA changes represented early stages of OA and mainly develop laterally. C57 black mouse was reported to develop OA mainly in the lateral side [15, 17], therefore, this study was consistent with these reports and that was why we investigated cytokine and HSP gene expression in lateral side of tibial plateau. Since C57 BL/6 mouse strain is closely related to C57 black mouse strain, it also developed early stage of OA changes (i.e., grade 1). The prevalence of OA changes in BL/6 mouse was about half that of C57 black mice.

Several cytokines have been known to regulate chondrocyte metabolism including proteoglycan synthesis and tissue degradation, however, this is the first study to show expression of IL-6 and IFN-γ mRNA in chondrocytes in early stages of primary OA in an experimental model. Furthermore, IFN-γ induction has been reported in synovial fluids of rheumatoid arthritis (RA) patients [30], although no induction of IFN-γ in OA has been reported. The high levels of IL-6 in synovial fluid have been reported in progressive OA in humans [31] and during initial processes of OA in a surgically induced experimental model [4]. Our results are consistent with these results, and indicate that one source of these cytokines in OA are cells of the articular cartilage.

IL-6 is a pleiotropic cytokine produced by cells of the immune and non-immune origin. Increased production of IL-6 is associated with disturbance of homeostasis, such as trauma, sepsis or inflammation [32–34]. Expression of IL-6 in human OA has been reported to be mediated by IL-1 [35], and blocking antibodies against IL-6 have been shown

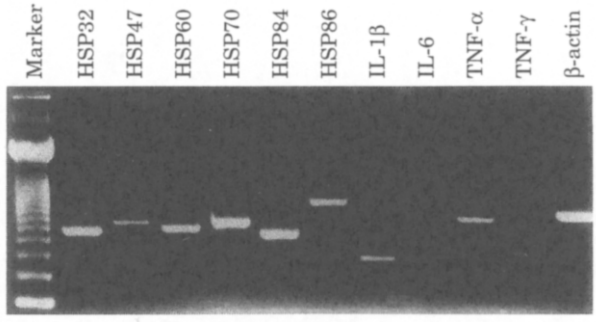

FIG. 3. RT-PCR products for HSP32, 47, 60, 70, 84, 86, IL-1β, IL-6, TNF-α, IFN-γ, and β-actin segments, specific to each cytokine and HSP were amplified from synthesized cDNA by PCR using the primers specific to each cytokine and HSP. All primer sets were designed to include at least one intron. This was done to ensure that PCR amplification from contaminating genomic DNA will not affect our observation.

Table III
Prevalence of cytokines and HSPs mRNA expression

Cytokine HSP	C57 black mice group as a whole $N=17$	C57 black mice group 8–12 months $N=7$	C57 black mice group 6 months $N=10$	BL/6 group 6 months $N=14$
HSP32	94%	86%	100%	93%
HSP47	29%*	71%‡§	0%	0%
HSP60	94%	100%	90%	86%
HSP70	82%*	86%‡	80%†	29%
HSP84	100%	100%	100%	100%
HSP86	89%*	86%	90%†	43%
IL-1β	100%	100%	100%	86%
IL-6	18%	43%‡	0%	0%
TNF-α	76%	86%	70%	43%
IFN-γ	88%*	100%	80%†	43%

Significant difference between *C57 black mice group as a whole and BL/6 group; †6 month old C57 black mice group and BL/6 group; ‡8–12 month old C57 black mice group and BL/6 group; §8–12 month old C57 black mice group and 6 month old C57 black mice group. *P*-values less than 5% were considered to be significant.

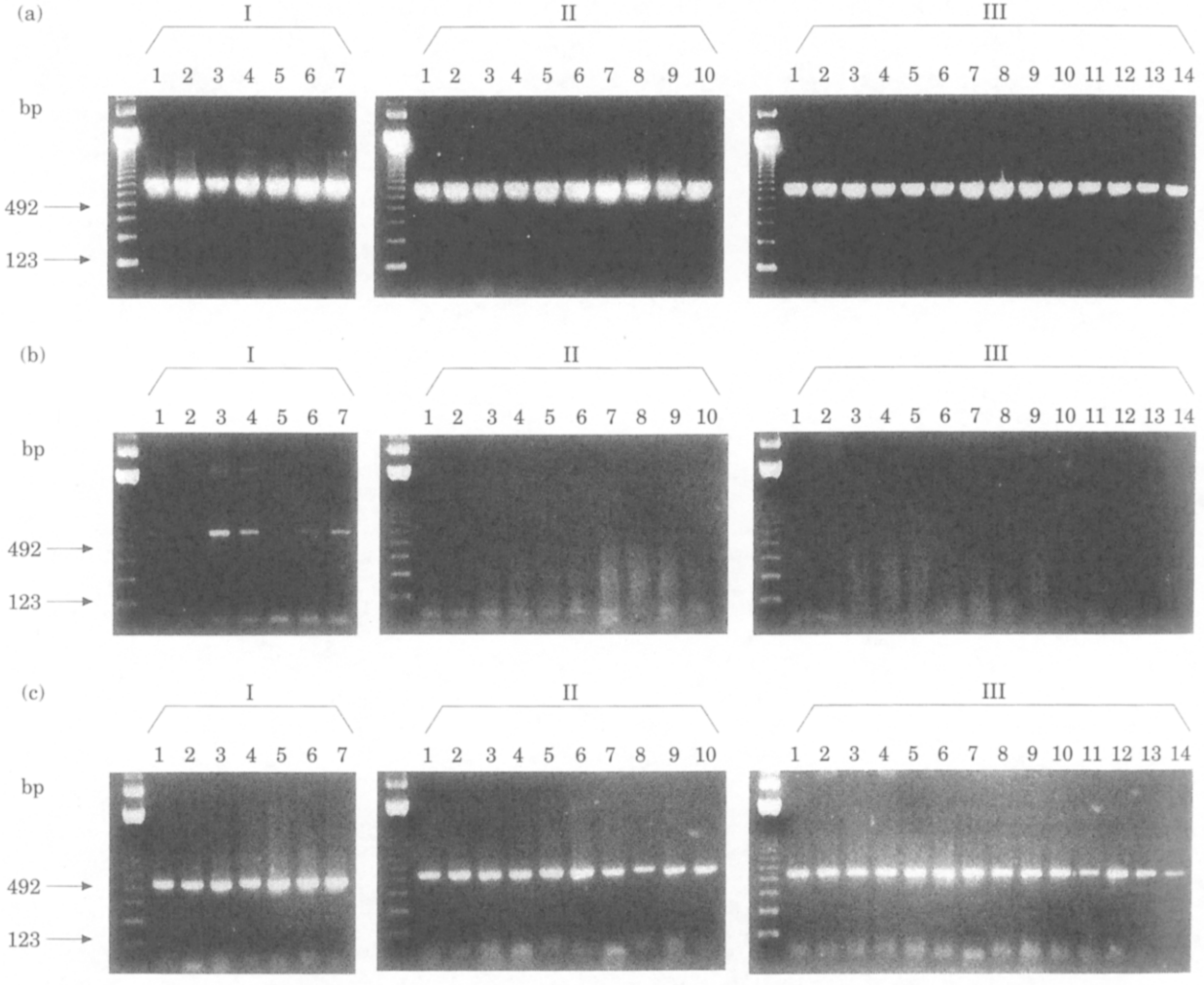

FIG. 4. Representative electrophoresis of RT-PCR. (a) β-actin mRNA expression in 8–12 month old C57 black mice group (I), 6 month old C57 black mice group (II), and BL/6 group (III). β-actin mRNA expression was detected clearly in all mice. (b) HSP47 mRNA expression in 8–12 month old C57 black mice group (I), 6 month old C57 black mice group (II), and BL/6 group (III). Five out of seven mice showed HSP47 mRNA expression in 8–12 month old C57 black mice group (lane 1, 3, 4, 6, and 7), while no expression was observed in 6 month old C57 black mice group and BL/6 group. (c) HSP84 mRNA expression in 8–12 month old C57 black mice group (I), 6 month old C57 black mice group (II), and BL/6 group (III). HSP84 mRNA expression was detected in all mice.

Table IV
Association of cytokines mRNA expression with HSPs mRNA expression

	IL-1β	IL-6	TNF-α	IFN-γ
HSP32				
HSP47		*		
HSP60	*			
HSP70			*	*
HSP84				
HSP86				*

Mutual association of cytokines and HSPs expression were analyzed statistically by 2X2 cross tables using Fisher's exact probability test. p values less than 1% were considered to be significant

to prevent IL-1 inhibition of proteoglycan synthesis in chondrocytes [36]. In this regard, our results are consistent in showing that IL-6 mRNA expressing mice always simultaneously expressed IL-1β and only high OA prevalence group expressed IL-6.

IFN-γ is a protein with varied effects that is involved in the mechanism of cells and organism defence against stress [37]. IFN-γ has been shown to induce class II major histocompatibility (MHC) antigens in chondrocytes. The chondrocytes that express MHC antigens stimulate lymphocyte proliferation [38]. Moreover, chondrocytes with MHC antigens express more stromelysin mRNA than chondrocytes without MHC [39]. Although, the biological effects of IL-6 and IFN-γ in chondrocytes in initial processes of primary OA remain to be defined, these cytokines are likely to play an important role in early stages of OA.

IL-1β and TNF-α are considered to be catabolic agents since they inhibit proteoglycan synthesis in cartilage [40] and stimulate chondrocytes to produce metalloproteinases [41, 42]. Previous studies have reported that both cytokines increase during OA [1–3]. We did not observe a significant difference between C57 black mice and BL/6 group with respect to the expression of IL-1β or TNF-α. We are aware that this could be a limitation of the highly sensitive RT-PCR technique used in this study. RT-PCR may maximally amplify even low levels of gene expression in the BL/6 group, thereby reducing our ability to discriminate relatively minor differences in the levels of gene expression between two groups with different prevalence of early stage OA.

In this study, the expression levels of HSP47, HSP70 and HSP86 were significantly higher in C57 black mice than in BL/6 mice. The expression of HSP32, HSP60 and HSP86 were detected in all animals. As mentioned earlier, the apparent lack of significant difference in the expression of HSP32, HSP60 and HSP86 between C57 black mice and BL/6 groups may be a reflection of the RT-PCR technique utilized in this study. We have previously demonstrated that chondrocytes from OA tissue enhanced HSP70 expression. The level of HSP70 increased with increasing severity of OA [8]. However, mechanisms of HSP enhancement in OA have not been clarified. Our data in this study imply that the expression of HSP70 correlates with the expression of TNF-α and IFN-γ (Table IV). Other studies have shown that HSP70 expression is induced by IL-1, and to a lesser extent, IL-2 and TNF-α [11–13]. In contrast, IFN-γ pre-treatment enhances HSP70 expression after a stress, although it does not induce HSP70 expression itself [43]. Heat treatment up-regulates the expression of HSP70 in TNF-α transfected cells [44].

HSPs either protect proteins from proteolytic degradation in a manner similar to chaperones or degrade damaged proteins. In particular, HSP47 is a specific chaperone for type I collagen [45]. In this study, HSP47 message was induced in C57 black mice in keeping with its role in regulating the cartilage phenotype.

In this study, we have evaluated the expression of HSPs and cytokines by articular cartilage in a spontaneous OA mouse model. We are aware that the OA cartilage may be composed of a heterogeneous population of chondrocytes, however, this fact does not alter the results obtained, since RT-PCR was performed on equal amounts of RNA obtained from whole tissue from all animals. We did not observe a significant correlation between HSP or cytokine expression and histological OA, perhaps because, in this study, we used the opposite side of the knees for histological analysis. Direct investigation of the localization of these factors by either *in-situ* hybridization or immunohistochemistry may reveal further detail.

In conclusion, this study demonstrated that HSP47, HSP70, HSP86, IL-6 and IFN-γ were expressed with greater prevalence in the cartilage of C57 black mice than the C57 BL/6 mice. These findings may suggest that chondrocytes are conditioned under non-physiological stress during early stages of OA. In addition, among HSPs, HSP70 was associated with two different highly expressed cytokines in C57 black mice, indicating the possible role of HSP70 as a indicator of early stage OA.

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