Human Keratinocytes *In Vivo* and *In Vitro* Constitutively Express The 72-kD Heat Shock Protein

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Exposure of cells to elevated temperatures induces a physiologic response characterized by the synthesis of a specific set of proteins (heat shock or stress proteins, HSPs) mediating repair mechanisms and protection from cellular damage. In the present study upon immunohistochemistry using a specific monoclonal antibody, the constitutive and heat-induced expression of the 72-kD HSP (HSP72) in normal human skin and in human epidermal cell lines (KB, A431) was investigated. Normal (unstressed) epidermis and adnexal structures of normal human skin were found to constitutively express HSP72. In contrast, a substantial HSP72 expression could not be observed in the dermal cellular compartment. *In vitro* heat treatment of punch biopsies from normal skin (42°C, 4 h) resulted in a further increase of epidermal HSP72 expression. In addition, dermal cells were found to be induced to express HSP72. To further evaluate the spontaneous HSP72 expression of epidermal cells two epidermoid carcinoma cell lines (A431, KB) were investigated. Upon immunohistochemistry and Western blot analysis a significant HSP72 expression could be detected in unstressed KB and A431 cells. In contrast, a human fibrosarcoma cell line (HT1080) was negative for HSP72 at 37°C but upon heat treatment a strong induction was observed. Furthermore, Northern blot analysis using a cDNA probe specific for human HSP72 revealed a constitutive expression of HSP72 mRNA in both epidermal cell lines. These findings demonstrate a significant expression of the stress-inducible HSP72 in unstressed human skin as well as in epidermal cell lines, suggesting that HSP72 may inherently be involved in the protective function of normal human skin. Key words: stress proteins/human skin/epidermis/heat. *J Invest Dermatol 101:334–338, 1993*

Heat shock proteins (HSPs) are induced by various cellular stress events and mediate a transient state of protection against subsequent cellular injury (e.g., thermotolerance) [1,2]. The 70-kD HSP family is one of the best-conserved stress proteins in the human system and consists of at least five different genes, coding for highly conserved "constitutive" and "inducible" proteins between 68 kD and 72 kD. Among these proteins recently the "inducible" 72-kD HSP (HSP72) has been described to play an important role in intracellular protein processing and to interact with cytokines and mediators such as IL-2 [3–5]. In contrast to certain cell lines (e.g., HeLa cells) in normal human tissues the constitutive expression of HSP72 has not yet been demonstrated [6]. However, recent studies have shown increased amounts of HSPs under pathologic conditions. Accordingly, chondrocytes of patients with osteoarthritis, hepatocytes from cirrhotic livers, and fibroblasts from patients with systemic sclerosis were reported to express HSP72 [7–9].

The epidermis is exposed to various environmental stress factors well known as inducers of a heat shock response *in vitro* [e.g., ultraviolet (UV) light, heat, mechanical alteration, microorganisms, drugs, and heavy metals]. To elucidate the significance of these proteins in human skin physiology recent studies focussed on the investigation of induction and regulation of HSPs in keratinocytes and skin fibroblasts [10–14]. Accordingly, upon stimulation with well-known inducers of a stress response, HSP72 was found to be expressed in human keratinocytes in cell culture as well as in an organ culture model [10,12,14]. However, the expression of HSP72 in normal human skin *in situ* has not yet been investigated. Therefore, in the present study the expression of HSP72 was analyzed in punch biopsies of normal human skin and in human epidermoid carcinoma cell lines.

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Abbreviations: HSP, heat shock protein; HSP72, 72-kD heat shock protein.

MATERIALS AND METHODS

Cell Culture, Skin Biopsy, Heat, and UVB Treatment The epidermoid carcinoma cell lines KB and A431 were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Ltd., Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS). The human fibrosarcoma cell line HT-1080 was grown in minimal essential medium (MEM; Gibco) supplemented with 10% FBS. *In vitro* heat treatment was performed by incubation of confluent cell cultures at 42°C for 4 h at 5% CO_2 in a humidified atmosphere. Samples of normal human skin were obtained from eight healthy volunteers by punch biopsy and snap frozen immediately. To exclude an induction of HSP72 expression during the biopsy procedure the time interval from subcutaneous injection of the local anesthetic (lidocain-HCl 2%) to freezing of the skin sample was less than 2 min. For *in vitro* heat treatment of normal human skin freshly obtained 2-mm punch biopsies were immediately immersed in serum-free DMEM supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and incubated at 37°C and 42°C, respectively. For UV treatment (20 mJ/cm²), a bank of four Westinghouse FS20 sunlamps was used. Constant temperature (37°C) during the irradiation period was maintained by a water bath. Cells were harvested for further analysis 4 h after UV exposure.

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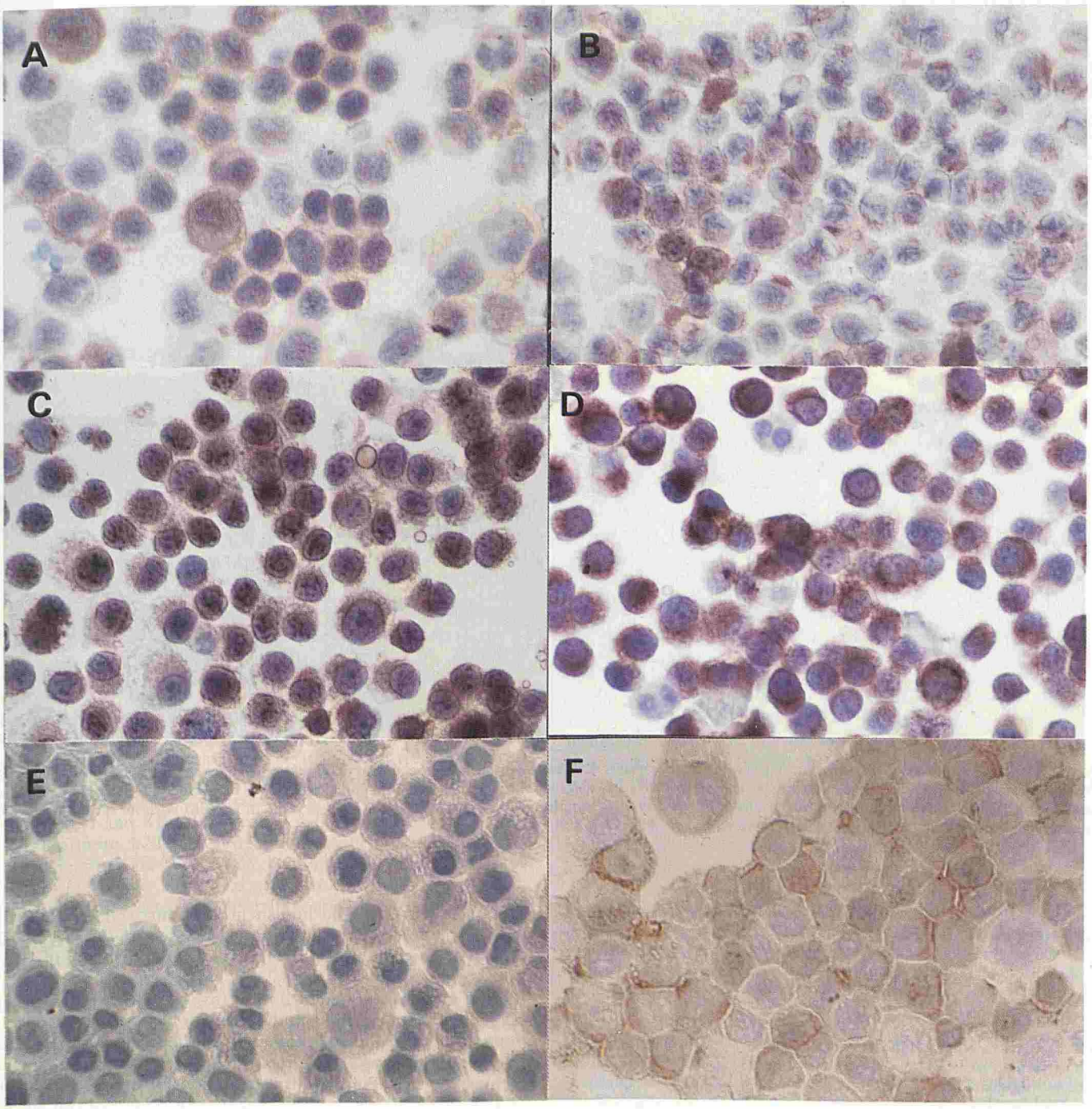

Figure 1. Immunohistochemical detection of HSP72 expression in epidermoid carcinoma cell lines (A431, KB) and a fibrosarcoma cell line (HT1080). *(A)* A431, 37°C; *(B)* A431, 42°C, 4 h; *(C)* KB, 37°C; *(D)* KB, 42°C, 4 h; *(E)* HT1080, 37°C; *(F)* HT1080, 42°C, 4 h. Counterstaining with hematoxylin, magnification ×250.

Immunohistochemistry For the detection of HSP72 in frozen sections of human skin and in cytospin preparations of cell lines a sensitive three-step immunoperoxidase technique was employed [15]. The fixed specimens were mounted with a commercially available monoclonal antibody specific for human HSP72 (Amersham, U.K.). An isotype-matched monoclonal control antibody was used for negative controls (anti-keyhole limpet hemocyanin, IgG_1; Beckton Dickinson, CA). Further incubations were done with peroxidase-conjugated rabbit anti-mouse and swine anti-rabbit immunoglobulin antibodies (Dakopatts, Copenhagen). 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) was used as a substrate, resulting in a red color. Counterstaining was done with Harris's hematoxylin (Merck, Germany).

Western Blot After incubation of cell cultures at the conditions indicated above culture media were discarded and cells were lysed by repeated cycles of freeze-thawings in purified water. The lysates were centrifuged and the supernatants were collected. Total protein content was measured by the method of Lowry *et al* [16]. Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed as described elsewhere [17]. Twenty micrograms total protein were loaded onto each lane of a 12% gel. After electrophoresis, proteins were transferred to nitrocellulose membranes by electroblotting [18]. Slot blots were performed for further quantification of HSP72 in whole cell extracts (Slot Blot Filtration Manifold, slot size 6 × 0.8 mm; Hoefer Scientific Instruments, San Francisco, CA). For each sample 20, 10,

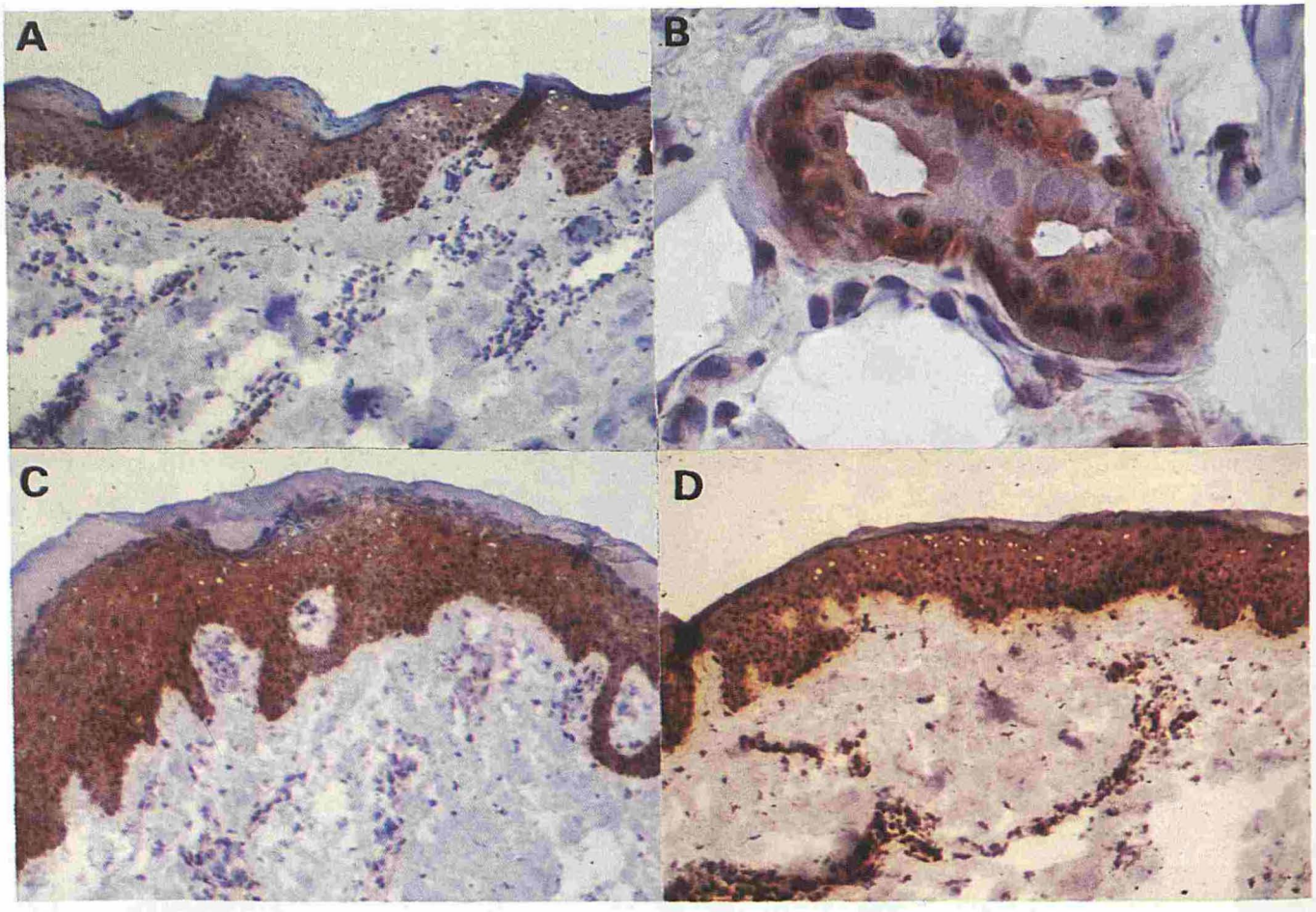

Figure 2. HSP72 expression in normal human skin (punch biopsies) detected by immunohistochemistry. *A)* skin sample was snap frozen immediately after biopsy without further incubation; *B)* eccrine sweat gland from freshly obtained skin sample; *C)* 2-mm punch biopsy was incubated for 4 h at 37° C; *D)* 2-mm punch biopsy was exposed to elevated temperature immediately after sampling (4 h, 42°C). Incubations were done in DMEM without serum at 5% CO_2. Counterstaining with hematoxylin, magnification *A,C,D* × 100; *B,* ×400.

and 5 μg total protein were loaded onto individual slots. Blots were incubated with the monoclonal antibody to HSP72 (Amersham). Bound antibody was detected by peroxidase-conjugated rabbit anti-mouse antibodies (Dakopatts, Copenhagen). 3-amino-9-ethylcarbazole (Sigma) was used as a substrate. Slot blots were evaluated by laser densitometry (Ultroscan XL; LKB, Sweden).

RNA Isolation and Northern Hybridization Northern blot analysis was performed according to standard procedures [19]. Total RNA was isolated by the guanidinium isothiocyanate method [20]. The samples were equalized for ribosomal RNA samples separated on 1% agarose gels. Equalized RNA samples (20 μg) were electrophoresed on 1% agarose/formaldehyde gels, followed by Northern transfer to nylon membranes (Sartolon, Sartorius, Germany). The cDNA probe specific for human HSP70 [20] was labeled with Klenow polymerase by random-primed incorporation of digoxigenin-labeled 2′-deoxyuridine 5′-triphosphate (dUTP) (Boehringer Mannheim Biochemica, Germany). Digoxigenin-dUTP–labeled nucleic acids were detected by a anti-digoxigenin alkaline phosphatase conjugate (Boehringer Mannheim Biochemica) and a chemiluminescent substrate (Lumigen PPD; Boehringer Mannheim Biochemica) according to the manufacturer's instructions. The membranes were exposed to X-ray film (Kodak XAR) for 30–60 min at room temperature.

RESULTS

Immunohistochemistry Significant amounts of HSP72 could be detected in KB and A431 cells under normal culture conditions (i.e., 37°C, 5% CO_2). Upon heat treatment (42°C, 4 h), a more intense cytoplasmatic and nuclear staining was observed in both cell lines (Fig 1*A–D*). The human fibrosarcoma cell line HT1080, which was used as a control, was negative for HSP72 at 37°C (Fig 1*E*). However, upon heat treatment a strong induction of HSP72 was observed (Fig 1*F*).

Skin samples from eight donors (five men, three women, ages 25–59) were obtained from different sites by punch biopsies. Immunohistology resulted in essentially the same expression pattern of HSP72 for all samples investigated. A representative experiment is demonstrated in Fig 2. In frozen sections of normal human skin HSP72 could be detected throughout the epidermal cell layers as well as in adnexal structures (e.g., eccrine sweat glands). No specific staining was observed in the dermal cellular compartment (Fig 2*A,B*). Upon *in vitro* heat treatment of normal human skin (42°C, 4 h) epidermal staining was intensified and a *de novo* expression of HSP72 in dermal cells was observed (Fig 2*D*). Control incubation of punch biopsies at 37°C for 4 h did not result in an induction of HSP72 in dermal cells. However, further induction of HSP72 in the epidermis upon incubation at 37°C cannot be excluded by immunohistochemistry (Fig 2*C*).

Western Blotting Western blot analysis was performed using A431 and KB cells. A specific band exhibiting a molecular weight of 72 kD was detected in extracts from KB and A431 cells under normal culture conditions as well as heat treatment or UV exposure (Fig 3*A–F*). In contrast, a specific band could not be detected in cellular extracts of HT1080 (Fig 3*G*). In contrast to epidermal cell

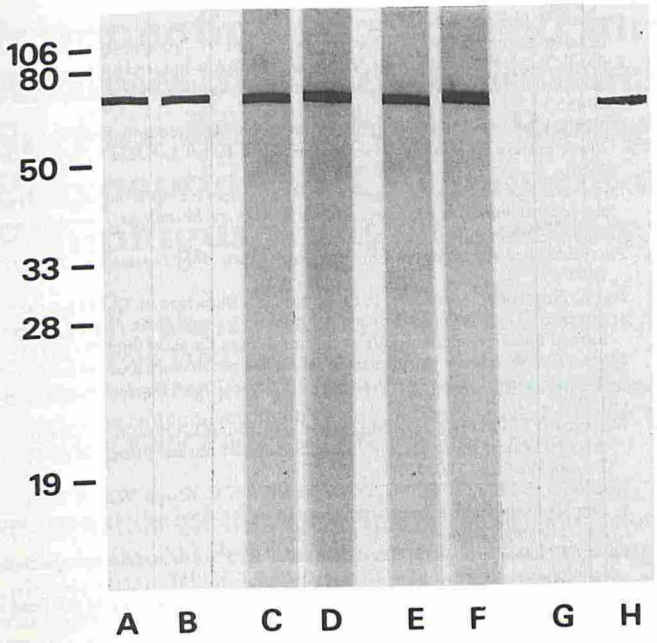

Figure 3. Expression of HSP72 in epidermoid carcinoma cell lines (A431, KB) and a fibrosarcoma cell line (HT1080) demonstrated by Western blotting. Cells were lysed by repeated cycles of freeze-thawing and 20 μg total protein were loaded onto each lane. Molecular weight markers (kD) are indicated to the *left. Lane A,* A431, 37°C; *lane B,* A431, 42°C, 4 h; *lane C,* KB, 37°C; *lane D,* KB, 42°C, 4 h; *lane E,* KB, 37°C; *lane F,* KB, 37°C, 20 mJ/cm² UVB; *lane G,* HT1080, 37°C; *lane H,* HT1080, 42°C, 4 h.

lines upon heat treatment a strong induction of HSP72 in the fibrosarcoma cell line (HT1080) was observed (Fig 3*H*). Further quantification of HSP72 induction was performed by titration of cell extracts on slot blots and subsequent laser densitometry (Table I). Opical densities (OD) of extracts (5 μg total protein) from unstressed A431 and KB cells were 0.2 ± 0.04 and 0.26 ± 0.03, respectively (mean $\pm$ SD). Heat treatment resulted in an 1.8 and 1.54 times increase of OD (A431: 0.36 ± 0.06, KB 0.40 ± 0.05). OD of HT1080 extracts at 37°C was at the background level (0.02 ± 0.01) and was increased to 0.43 ± 0.04 upon heat treatment.

Northern Blotting To investigate HSP72 expression in epidermoid cell lines at the mRNA level Northern blot analysis was performed using a specific cDNA probe. Unstimulated A431 and KB cells constitutively expressed HSP72-specific mRNA (Fig 4, *lanes 1,3*). Upon heat treatment a strong induction of HSP72 mRNA expression was observed (Fig 4, *lanes 2,4*). The mobility of the detected mRNA corresponded to the reported size of the HSP72 transcript (2.3 kB) [21]. In the control cell line (HT1080) constitutive expression of HSP72 mRNA was at the sensitivity limit of our detection system. In accordance with Western blot analysis heat treatment induced a strong induction of HSP72 in these cells also at the mRNA level (Fig 4, *lanes 5,6*).

Table I. Induction of HSP72 upon Heat Treatment Determined by Laser Densitometry of Slot Blots[a]

	A431	KB	HT1080
37°C	0.20 ± 0.04	0.26 ± 0.03	0.02 ± 0.01
42°C	0.36 ± 0.06	0.40 ± 0.05	0.43 ± 0.04
Induction[b]	1.8	1.54	21.5

[a] Epidermal cell lines (A431, KB) and the fibrosarcoma cell line (HT1080) were incubated at the indicated temperatures for 4 h. Whole cell extracts were equalized for total protein content and loaded on slot blots. HSP72 was detected by a monoclonal antibody as described in *Materials and Methods.* Slots containing 5 μg total protein were analyzed by laser densitometry. Samples were analyzed in triplicate. Values represent mean $\pm$ SD of OD values.

[b] Induction was defined as OD of samples incubated at 37°C divided by OD of heat-treated samples.

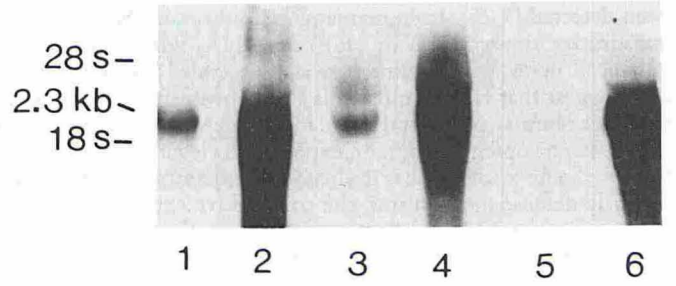

Figure 4. Expression of HSP72 mRNA in epidermoid carcinoma cell lines (A431, KB) and a fibrosarcoma cell line (HT1080) demonstrated by Northern hybridization. 20 μg of total RNA were loaded onto each lane and hybridized against HSP72 cDNA. Mobilities of 18S and 28S rRNA and mRNA size are shown to the *left. Lane 1,* A431, 37°C; *lane 2,* A431, 42°C, 4 h; *lane 3,* KB, 37°C; *lane 4,* KB, 42°C, 4 h; *lane 5,* HT1080, 37°C; *lane 6,* HT1080, 42°C, 4 h.

DISCUSSION

The results of this study demonstrate that the stress-inducible HSP72, a major member of the highly conserved 70-kD HSP family, is constitutively expressed in normal human skin and in cell lines derived from epidermal tumors. The 70-kD HSPs include a complex family of both constitutively expressed and stress-inducible genes. The constitutively expressed 70-kD HSPs (hsc genes) are detectable in a variety of cells under physiologic conditions and are not induced by stress factors. In contrast, the HSP72 gene product is strongly induced by heat and other stress events and is not detectable in significant amounts in unstressed cells. Recently, it was reported that members of the 70-kD HSP family contribute to intracellular protein processing [5]. They are able to recognize and stabilize partially folded intermediates during polypeptide folding, assembly, and disassambly under physiologic conditions as well as at elevated temperatures. Furthermore, these proteins appear to play a role in antigen processing [22].

Recent investigations demonstrated that HSP72 can be induced in cultured human dermal fibroblasts and in keratinocytes upon treatment with heat, heavy metals (e.g., Cd^{++}), toxic agents (e.g., arsenite), and cytotoxic prostaglandins [10,12]. In organ-cultured human skin, ultraviolet light (UVB, UVC) also was found to be a potent inducer of HSP72 expression in keratinocytes [14]. However, in this study a constitutive expression of HSP72 in human skin explants could not be detected. This discrepancy may be due to different techniques, in particular to a higher sensitivity of our three-step immunohistochemistry, compared to indirect immunofluorescence. The constitutive expression of HSP72 may be found to be below the threshold of the technique used by Muramatsu and co-workers. In the present study human epidermal cells *in vivo* and *in vitro* were found to constitutively express HSP72. Similarly, constitutive HSP72 expression by epidermal cells in culture has recently been reported [23]. In this study no upregulation of HSP72 mRNA could be induced by UV exposure. However, so far no data on the expression of HSP72 in freshly obtained human skin have been available. In other human organs it has been shown that under some pathologic conditions HSP72 expression is induced. Accordingly, HSP72 expression has been detected in cirrhotic livers and in chondrocytes from patients with osteoarthritis [8,9]. In liver biopsies, as well as in chondrocytes from healthy donors, HSP72 is not detectable by immunohistology. In addition, HSP72 is expressed in neutrophils from patients suffering from severe tissue injury, but not in neutrophils from healthy donors [24]. In scleroderma fibroblasts and peripheral blood mononuclear cells from systemic lupus erythematosus patients elevated transcription rates for HSP72 have

been detected [7,25]. In human epidermoid carcinoma cell lines a constitutive transcription of HSP72 mRNA, which was further enhanced upon heat treatment, was detectable. Similarly, recent data suggest that HSP72 mRNA is also constitutively expressed in unstressed human peripheral blood monocytes and neutrophils [26], which can be upregulated upon exposure to elevated temperatures. There is some evidence that in these inflammatory cells that mediate early defense mechanisms, the constitutive expression of stress proteins may render the cells more resistent to the hostile microenvironment of inflammatory foci. Similarly to peripheral blood inflammatory cells, the expression of HSP72 in epidermal cells may be necessary to maintain the protective function of human skin and to mediate their resistence against temperature changes, mechanical alterations, UV light, drugs, and toxic molecules.

The abundance of HSP72 in human epidermis and in epidermal cell lines, in association with its possible role in intracellular protein translocation, protein folding, antigen processing, and protection from thermic, actinic, and oxidative injury, suggests a significant role for this protein in skin physiology. Investigations on the expression and function of HSPs in skin diseases may give a further insight into the biologic role of the heat shock response. The expression of HSPs in these pathologic conditions may be of importance for therapeutic regimens including hyperthermic perfusion, UV, photochemotherapy, and photodynamic treatment [27].

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