



Overexpression of murine small heat shock protein HSP25 interferes with chondrocyte differentiation and decreases cell adhesion

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Abstract

Although multiple functions for the small heat shock protein HSP25 have been proposed, its specific role during developmental and differentiation processes is not known. Cartilage is one of the tissues in which HSP25 is specifically and highly expressed during development. C1 cells, able to form aggregates *in vitro*, can be induced to differentiate into chondrocytes. In this study, we generated two stable transfected clones overexpressing HSP25 at two different levels. Cell morphology and growth rate were modified in both clones, although the actin content and distribution did not seem to be altered. Overexpressing clones had more difficulties in coalescing, leading to smaller aggregates and they did not differentiate into chondrocytes. Subsequently, these aggregates tended to dissociate into loose masses of dying cells. The strength of all these effects was directly correlated to the level of HSP25 overexpression. These data suggest that overexpressing HSP25 decreases cellular adhesion and interferes with chondrocyte differentiation.

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Keywords: small heat shock proteins; HSP25; cell adhesion; chondrocyte differentiation; cell death

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; GSH, reduced glutathione; PBS, phosphate buffer saline; SDS, sodium dodecyl sulfate; SSC, standard saline concentration

Introduction

HSP25 (called HSP27 in human cells) belongs to the small heat shock protein family. Members of this protein family share similar structural features: a small molecular size (20–

30 kDa) and a highly conserved carboxy terminal region, called the α -crystallin domain.¹ HSP25 was the first small heat shock protein to be described and its synthesis is highly induced in response to heat shock as well as chemical and oxidative stresses. α A- and α B-crystallin were initially described as eye lens structural proteins. Yet, α B-crystallin shares similar function and sequence with HSP25. It can be induced by heat, chemical and oxidative stresses. The roles of these proteins as chaperones are well documented.² The HSP25 distribution pattern has been studied in both adult mice and during the embryonic development.^{3–7} It appears to be highly expressed in specific organs or tissues such as the heart,⁸ skeletal striated muscles, epidermis, cartilage, bone⁹ and specific areas of the central nervous system.^{10,11} But nothing is known about how the possible role of this protein may explain such a specific distribution.

Several roles have been proposed for HSP25 from *in vitro* studies. It is well documented that it can interfere with actin polymerization and stabilization of microfilaments^{12–14} and HSP25 phosphorylation (through the p38 kinase pathway) is necessary for stress fibers and actin filament dynamics regulation.^{15–20} It could also interact with intermediate filaments and microtubules,^{21–23} although no direct proof has been given. This is in good agreement with the morphological modifications and changes in the cell proliferation rates observed in several cellular systems where this protein is over or underexpressed.^{24–26} Another role has been proposed by the laboratory of Dr. A-P Arrigo: it could act as a switch between differentiation and apoptosis as demonstrated during cell line differentiation *in vitro*.^{27,28} This role has been correlated with reduced glutathione regeneration in response to stress.^{29,30} But the cellular mechanism is not clearly identified yet for the action of HSP25 in the differential control of differentiation and apoptosis.

We were interested in understanding mechanism of HSP25 action at the cellular level and during the physiological process of differentiation. Our work has focused on cartilage differentiation. Cartilage is one of the tissues where HSP25 is most highly expressed apart from in the heart and skeletal muscles, both in the adult mouse and throughout embryonic development.¹⁰ HSP25 quantity increases during chondrocyte maturation, but it is more weakly expressed in the bone.

In order to determine the role of HSP25 in this differentiation process, we aimed at modifying its expression through overexpression and evaluated the effects of this overexpression on chondrocyte differentiation. Chondrocyte maturation is usually studied using mesenchymal dissected explants, kept in culture. The disadvantage of such a system is that it consists in a mixture of different cell types. We used another system: the C1 cell line. This

teratocarcinoma-derived cell line^{31,32} can, *in vitro*, be specifically differentiated into chondrocytes, osteocytes or adipocytes according to the inducer used.³³

We have stably transfected C1 undifferentiated cells with an expression vector containing the complete HSP25 cDNA, selected two HSP25 overexpressing clones and studied the cartilage differentiation capacities of these clones. Our results show that HSP25 overexpression disturbs cell adhesion resulting in the alteration of chondrocyte differentiation in the C1 cells.

Results

Screening of HSP25 overexpressing clones

In order to obtain HSP25 overexpressing clones, C1 cells were transfected with an expression plasmid carrying the complete HSP25 cDNA sequence, in sense orientation, under the control of the SV40 promoter and polyadenylation signal (see Material and Methods). A second plasmid was co-transfected for hygromycin resistance selection. After 3 weeks, hygromycin resistant clones could be detected. Seventeen of them, named sense clones, S, were picked and further sub-cloned. Figure 1A displays the results of a Western blot conducted on protein extracts from nine independent clones. S1-1, S4-2, S5-2, S5-3, S8-3 correspond to clones transfected with the HSP25 cDNA containing plasmid. C1 are untransfected control cells. P1-1, P4-3, P6-8, P8-2 correspond to C1 cells transfected with both the hygromycin resistance plasmid and the empty expression

vector pSVK3 with no HSP25 insert. No HSP25 protein can be detected in proliferating untransfected C1 cells (lane 2), or in cells transfected with the empty vector (P clones: lanes 3, 4, 5, 6). This indicates that HSP25 is barely, if at all, expressed in proliferating C1 cells. Two highly overexpressing clones are detected: S4-2 (lane 8) and S5-3 (lane 11). The HSP25 content of S4-2 is slightly higher than that of S5-3. Further quantitation has been conducted on Western blots, comparing HSP25 content in S4-2 and S5-3 protein extracts, to known amounts of pure HSP25 protein (data not shown). S4-2 contains about 250 ng HSP25/mg total protein whereas S5-3 content is about 150 ng/mg total protein. The expression level in clone S4-2 is thus 1.6 times higher than in S5-3. No protein was detected in the C1 extract, meaning those cells contained less than 10 ng/mg total protein. HSP84 protein content, used as an internal control, was not modified in HSP25 overexpressing clones, indicating no global HSP alteration.

Northern-blot analysis of RNA extracted from proliferating S4-2 and S5-3 cells has confirmed these results (Figure 1B). No HSP25 RNA could be detected in C1 cells (lane 1), or in P clones extracts (lanes 4 and 5); therefore transfection in itself does not affect HSP25 expression. In contrast, two major bands are revealed in S4-2 and S5-3 cells (lanes 2 and 3). The upper band corresponds to the expected size of the transgene RNA (about 1900 nt) using the SV40 polyA signal. The lower band corresponds to the size of a transcript which uses the polyA signal present at the 3' end of the HSP25 cDNA insert. It leads to a transgene transcript of about 1000 nt. Both bands display higher molecular size than the endogenous HSP25 mRNA, which is about 900 nt long^{34,35} as seen in the F9 cell extract (lane 6) in which HSP25 is constitutively expressed. A third, smaller (less than 900 nt) and fainter, signal is detected in clone S4-2. It might correspond to the use of an internal transcriptional initiation signal and lead to a partial transcript.

Thus, HSP25 protein overexpression observed in S4-2 and S5-3 clones (Figure 1A, lanes 8 and 11) is correlated with high amounts of HSP25 transgene RNAs.

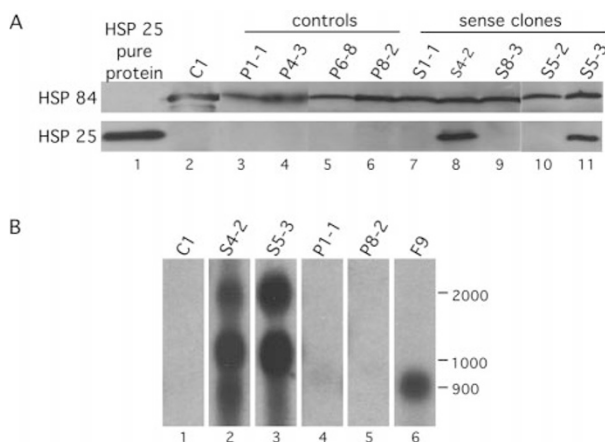


Figure 1 HSP25 levels in overexpressing clones. **(A)** HSP25 protein is overexpressed in two stably transfected clones. Ten μ g protein from each cell extract was used in HSP25 and HSP84 immunoblot analyses of control untransfected C1 cells (lane 2), C1 cells transfected with the pSVK3 empty plasmid (P1-1, P4-3, P6-8, P8-2: lanes 3–6) or with the HSP25 containing plasmid (sense clones: lanes 7–11). Lane 1 contains 100 ng purified HSP25 protein (StressGen, Canada). **(B)** HSP25 protein levels are correlated with transcription of the transgenes. Twenty μ g of total RNA was loaded in each lane and resolved by 1% agarose gel electrophoresis. A cDNA probe, mapping to the 5' part of the HSP25 transcript, was used for Northern blot analysis. The appropriately sized band was detected in clones overexpressing HSP25 (S4-2 and S5-3) but not in nontransfected cells (C1) or C1 containing vector alone (P1-1, P8-2). For comparison, lane 6 contains RNA from F9 cells, which constitutes a positive control for HSP25 endogenous transcription. Approximate size of the bands is indicated in nucleotides

HSP expression in proliferating cells of control and HSP25 overexpressing clones

HSP25 distribution in C1 control and overexpressing cells was examined by immunofluorescent staining of fixed cells (Figure 2). In C1 cells, despite the absence of a signal in Western and Northern-blot analyses, a faint staining can be detected in the cell cytoplasm (Figure 2a). In S4-2 (Figure 2e) and S5-3 (Figure 2i) cells, a strong signal is detected. It is uniformly distributed in the cytoplasm with no nuclear coloration. No protein aggregates could be detected even when observed at higher magnification. Thus, overexpression of HSP25 in S4-2 and S5-3 clones does not appear to alter the global cellular distribution of this protein and does not lead, in our case, to macroscopic HSP25 aggregates.

HSP84, used as control, as well as other small HSPs (α B-crystallin, MKBP) expression patterns are not modified by HSP25 overexpression. HSP84 (Figure 2b,f,j) displays a specific perinuclear localization in highly colored bubble shaped compartments. α B-crystallin staining is similar in all

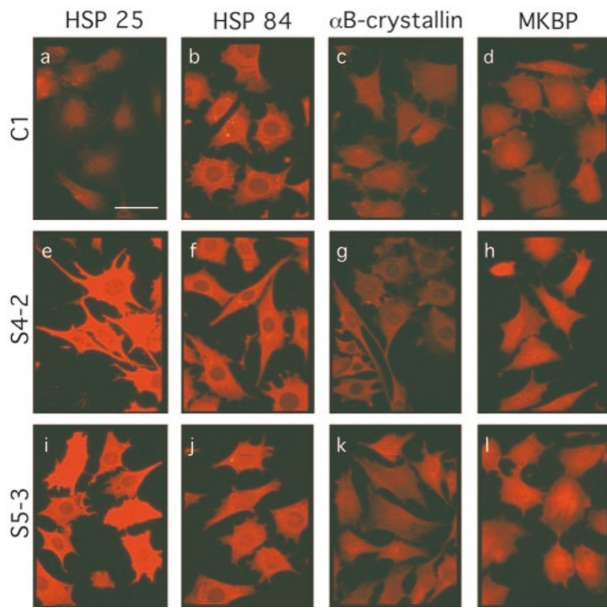


Figure 2 HSP25 overexpression does not modify the expression of other HSPs in proliferating cells. Proliferating cells of C1 (a,b,c,d) and HSP25 overexpressing clones S4-2 (e,f,g,h) and S5-3 (i,j,k,l) were fixed and immunolabeled with anti HSP25, anti HSP84, anti α B-crystallin, anti MKBP, followed by appropriate cy3-linked secondary antibodies. Though the level of HSP25 in clones S4-2 and S5-3 is increased overall (e,i), it remains predominantly cytoplasmic. The expression level and the distribution of related small HSPs, α B-crystallin (c,g,k) and MKBP (d,h,l), and another HSP, HSP84 (b,f,j), are not altered by HSP25 overexpression. Bar: 40 μ m

three clones and close to what can be seen in C1 cells for HSP25 (Figure 2c,g,k). MKBP is a third member of the smHSPs family, primarily described in the heart tissue.³⁶ Its expression is homogenous (Figure 2d,h,l). These results underline the specificity of HSP25 overexpression.

Cell proliferation in control and HSP25 overexpressing clones

HSP25 overexpression seems to be correlated, in some transfected cellular systems, with reduced (A-P Arrigo, personal communication) or increased³⁷ proliferation rate. In order to estimate the length of the cell cycle, we analyzed growth kinetics. The three clones of interest, C1, S4-2 and S5-3, were initially plated at similar densities, then harvested and counted in triplicate, for the following 9 days. Growth curves are illustrated on Figure 3. Statistic comparison (Anova test) of the exponential phase of the growth curves shows that S4-2 and S5-3 doubling times are statistically different from that of C1 ($P=0.001$) and are about 22 and 19 h, respectively, whereas the doubling time of C1 is 14 h. Thus, both HSP25 overexpressing clones display reduced (up to 57%) proliferation rates when compared to untransfected C1 cells. The amplitude of the decrease is correlated with the level of HSP25 overexpression (S4-2: 57%; 250 ng/mg and S5-3: 35%; 150 ng/mg).

Morphological alterations can also be detected in HSP25 overexpressing clones. C1 cells are polygonal cells and are generally quite large and flat (about 40 μ m diameter)

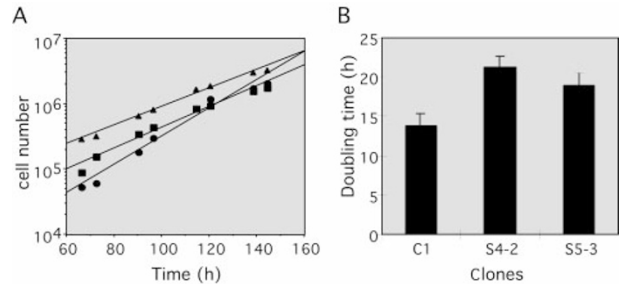


Figure 3 Proliferation rate is reduced in HSP25 overexpressing clones. (A) Semi-logarithmic representations of cell number versus time of culture (in hours). Initial seeding was at 10⁵ cells/6 cm tissue culture dish. Each point corresponds to the mean of a triplicate. \blacktriangle S4-2 \blacksquare S5-3 \bullet C1 cells. (B) The slope of the curves obtained in A was used to calculate the mean and standard deviation of the cells doubling times. The doubling time was significantly increased in clones S4-2 and S5-3 compared to parental C1 cells

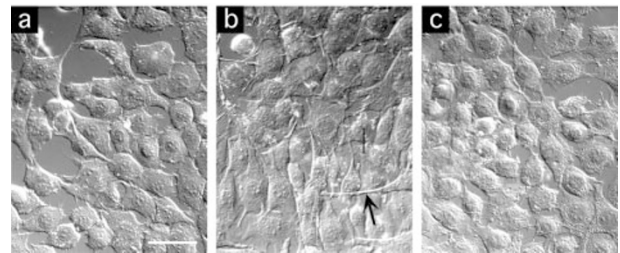


Figure 4 Morphology proliferating C1 cells and HSP25 overexpressing S4-2 and S5-3 clones. Microscopic images taken using Nomarski interference contrast of C1 (a), S4-2 (b) and S5-3 (c) half confluent cells demonstrate the altered phenotype of S4-2 and S5-3 cells. The arrow indicates one of the thin prolongations typical of S4-2 cells. White bar: 50 μ m

(Figure 4a). S4-2 cells were much longer and thinner with a fibroblast-like morphology (see Figures 4b and 2e–h). S5-3 displayed an intermediate phenotype.

Actin filaments in control and HSP25 overexpressing cells

The previous observations and literature data led us to compare HSP25 localization with that of some cytoskeleton components. Cellular actin filaments can easily be visualized using fluorescein-linked phalloidin. No difference could be detected between the different clones in their actin network, either in the distribution of the filaments, or in their lengths: overexpression of HSP25 does not seem to globally disturb the actin filament network in these cells (Figure 5b,e,h). When the actin and HSP25 signals were superimposed (Figure 5c, f,i), we observed an overlapping signal (yellow) in S4-2 and S5-3 clones. The predominant green signal in C1 cells (Figure 5c) reflects their low HSP25 content.

Chondrogenic differentiation of control and HSP25 overexpressing clones

C1 cells have been described as a practical system to study chondrocyte differentiation. Upon dexamethasone addition, cells differentiate into proliferating, then mature and

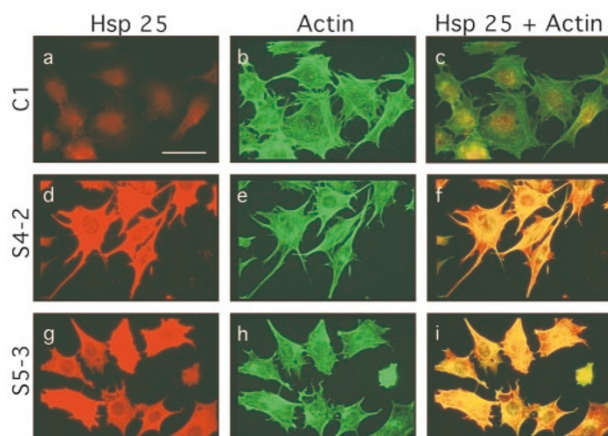


Figure 5 The actin filamentous network and HSP25 distribution overlap in HSP25 overexpressing S4-2 and S5-3 clones. Proliferating cells of C1, S4-2 and S5-3 clones were fixed and labeled with FITC-linked phalloidin and anti HSP25 followed by a cy3-linked secondary antibody. Double exposure of cy3 and FITC fluorescence (c,f,i) demonstrates the colocalization of HSP25 and actin. Bar: 40 μm

subsequently hypertrophic chondrocytes, expressing *in vitro* several cartilage specific markers such as collagen II and collagen X.^{31–33} This differentiation process requires an initial aggregation step (9 days named d-9 to d0) when cells proliferate and form spherical aggregates. These aggregates continue growing in size during the first 20 days of differentiation but begin to differentiate after inducer treatment (dexamethasone is added at d0).³³ All analysis were performed on aggregates harvested at four different stages: day 0 (d0=induction), 12 (d12), 26 (d26) and 48 (d48).

Formation of the aggregates

During the initial aggregation step (from d9 to d0), we noticed that HSP25 overexpressing cells had difficulties in forming aggregates. Figure 6A shows the detailed size distribution of the aggregates, estimated at d0 (see Materials and Methods): the radius of C1 aggregates covers a large range of sizes, reaching values of over 125 μm (Figure 6A, a); the size distribution of S4-2 and S5-3 also covers a wide range, but never exceeds 90 μm for S5-3 (Figure 6A, c) and hardly reaches 80 μm for S4-2 (Figure 6A, b). The mean radius of the aggregates formed from S4-2 and S5-3 cells was smaller than the mean radius of those formed by C1 cells: while C1 aggregates had an average radius of 58 μm , the average radius was 20% and 38% lower for S5-3 (46 μm) and S4-2 (36 μm), respectively (Figure 6B). Statistical analysis (*t*-test) revealed these differences to be highly significant (Figure 6C). It should be noticed that the difference in cell growth rate mentioned above cannot account for the differences in the size of the aggregates. Indeed, when the starting densities are increased, the number of aggregates increases, but their average size remains the same (data not shown).

Between d0 and d12, S4-2 aggregates completely dissociated. Only clone S5-3 could be followed in differentiation until d48. Thus, most of the following data only concern C1 and S5-3 clones.

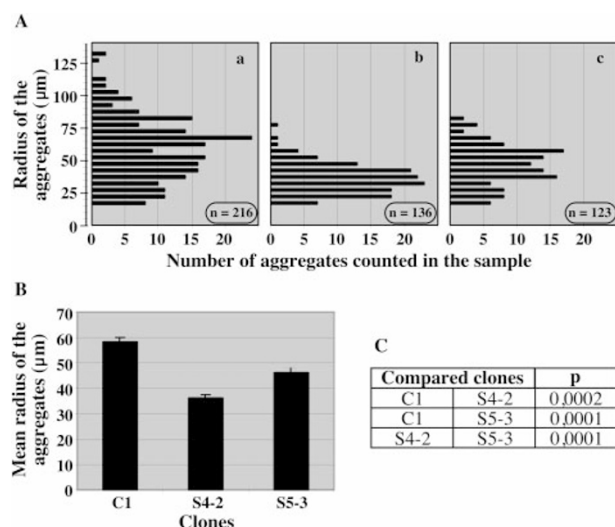


Figure 6 Comparison of the size of cellular aggregates at the end of the compaction phase. At day 0, aggregates were collected and their radii calculated as described in Materials and Methods. (A) The size distributions, in terms of radius, established for C1 (a), S4-2 (b) and S5-3 (c) aggregates indicate a reduced proportion of large aggregates in the clones expressing HSP25. (B) Mean radius and standard error calculated, for each clone, from the data above. (C) The results of the statistical test (*t*-test) show a significant size difference between each of the three clones

Differentiation markers

The differentiation of cartilage was followed by analyzing the expression of collagens I and II, two main markers of this differentiation (Figure 7). Collagen I is a specific marker of bone, absent from the mature and hypertrophic chondrocyte stages, but known to be expressed in chondroprogenitor cells.³⁸ As for collagen II, it is a specific marker of mature and hypertrophic chondrocytes. Our results of Northern-blot analysis of C1 aggregates at d0, 12, 26 and 48 (Figure 7A) confirm previous observations.³³ Collagen I expression, already significant at d0 (lane 1) is maximum at d12 (lane 2), then decreases at d26 and d48 (lanes 3 and 4). Collagen II expression, in contrast, is weak at d0 (lane 1), increases until d26 and then remains high. In S5-3 aggregates, collagen I and II expression appeared similar to that observed in C1 clone at d0 (lane 5). But their expression is drastically reduced as early as d12, both for collagen I and collagen II (lane 6). The latter does not increase as expected in normal differentiation and is totally absent at d26 and d48 (lanes 7 and 8). This clearly demonstrates that expression of collagen markers was altered in HSP25 overexpressing cells. The corresponding protein expression was examined by immunocytochemistry of sections of the aggregates. This is represented in Figure 7B for the cartilage specific collagen, collagen II. These observations support the results of the Northern blot. Collagen II expression increased in C1 aggregates until d26 and then decreased (Figure 7B, a–d). In S5-3 aggregates, it was at a low level at d0, it remained faint at d26 and then completely disappeared (Figure 7B, e–h). Alcian blue staining was also performed on aggregate sections. This dye colors sulfated glycosaminoglycans of the extracellular matrix and is thus specific to cartilage matrix.

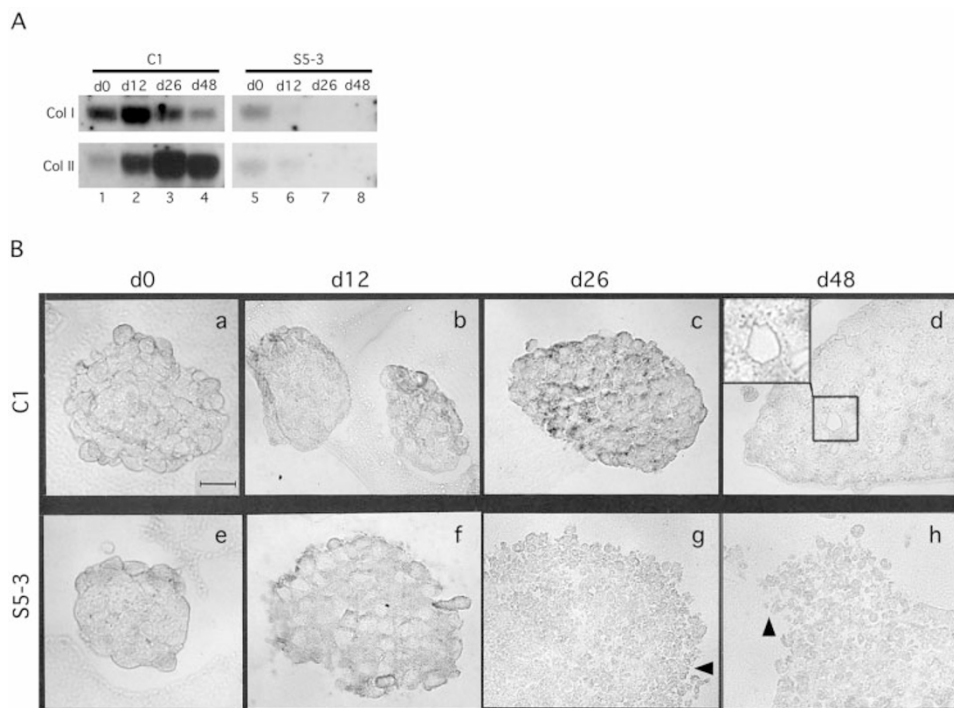


Figure 7 Collagen I and II expression in C1 aggregates is characteristic of chondrocyte differentiation whereas they are altered in HSP25 overexpressing S5-3 clone. **(A)** Specific cDNA probes were used for Col I or Col II detection in Northern-blot experiments. During the differentiation of C1, tested at the time points indicated, mRNA for collagen I appears transiently with a peak at d12, and collagen II appears progressively until d26 and decreases afterwards (lanes 1–4). In contrast, little signal was detected in differentiating S5-3 (lanes 5–8). **(B)** C1 (a–d) and S5-3 (e–h) aggregates harvested at indicated timepoints were fixed, embedded in paraffin and sections labeled with anti-collagen II antibody. During the differentiation, the appearance of collagen II protein in the C1 aggregates was also progressive until d26. It was reduced at d48. Little collagen protein was detected in S5-3. The inset in (d) shows the detail of a lacuna (2 × enlargement with enhanced contrast). Arrowheads indicate the dissociation of cells on the edge of S5-3 aggregates at d26 and d48

A blue coloration appears as early as d26, a stage which corresponds to chondrocyte maturation and to the expression of collagen II. Staining continues at later stages when the extracellular matrix is more abundant, surrounding a few living cells in lacunae. With S5-3 aggregates, no blue coloration could be seen in the first stages (d0, d12) and further dissociation of the aggregates did not allow confident staining of aggregates (data not shown).

Collagen I and II expression and Alcian blue staining in C1 aggregates all confirm previous *in vitro* observations in the C1 system and *in vivo* kinetics of cartilage differentiation. Therefore, the alteration observed with S5-3 clone for those two aspects of the differentiation tend to indicate that cartilage differentiation was altered by HSP25 overexpression.

Morphology of the aggregates

Characteristic morphological changes can be noticed during the cartilage differentiation process of C1 cells. Aggregates first appear as homogenous, compact, cell gatherings. Cell division ceases around day 26 (d26) when chondrocytes become mature. Chondrocytes then increase in size becoming hypertrophic chondrocytes and secrete abundant extracellular matrix. Lacunae begin to form, surrounded by abundant matrix and including rare living cells. This characteristic aspect of C1 aggregates at d48 can be seen

in Figure 7B, d. Morphological aspects of the aggregates therefore constitute good indicators of the differentiation process. It should also be mentioned that even the C1 aggregates which were of a size comparable to the mean size of S5-3 aggregates had this morphology.

As noticed above, from immunological observations (Figure 7B), the morphology of S5-3 aggregates seems to be altered : they look like C1 aggregates at the early stages (d0, d12), with similar shapes and cell boundaries (Figure 7B, e–f). By d26, HSP25 overexpression leads to less cohesive aggregates which tend to disassemble and display more diffuse cellular boundaries; also, nuclei were not as clearly distinguishable (Figure 7B, g–h). Furthermore, the classical morphology of hypertrophic cartilage as detected in C1 cells was never observed in HSP25 overexpressing clones. No lacunae and no embedding of large cells in extracellular matrix could be seen (compare Figure 7B, d,h).

The analysis of the shape of whole aggregates (Figure 8) led to similar conclusions : S5-3 aggregates are shaped like C1 aggregates until d12 (Figure 8a,b,e,f), even though they remain smaller (see Figure 6); then they become loose masses of dissociating cells (Figure 8c,d,g,h). This modified morphology was observed in all S5-3 aggregates but never, at any stage, in C1 aggregates.

We mentioned above that S4-2 aggregates were completely dissociated as early as d12. Thus, the different

observations for S4-2 and S5-3 may be accounted for by a change in adhesive properties that would occur sooner in S4-2 which expresses a higher level of HSP25.

In order to clarify whether these morphological changes were correlated with cell death, we observed nuclei after Hoechst coloration of C1 and S5-3 aggregates throughout the differentiation process (Figure 9). Nuclei appeared with clear limits in C1 aggregates at all stages of differentiation (Figure 9a–d). The aggregates initially appeared compact (d0, d12) whereas nuclei progressively appeared more distant from one another reflecting the increase in cell spacing due to extracellular matrix deposition (d26, d48). The nuclei do not seem to be modified in the early stages of differentiation (d0 : Figure 9e) in S5-3 aggregates, even though the aggregates did not contain as many cells as C1 aggregates. But nuclear integrity was severely affected by stage d26 when nuclei could hardly be seen. The Hoechst reagent colored small spots distributed in the whole aggregate (Figure 9g). This phenomenon was further accentuated at d48 when ever fewer intact nuclei were detected (Figure 9h). Nuclear integrity was thus altered as early as d12 in S5-3 cells and this continued until d48. This demonstrates that the cells remaining in S5-3 aggregates at d26 are probably

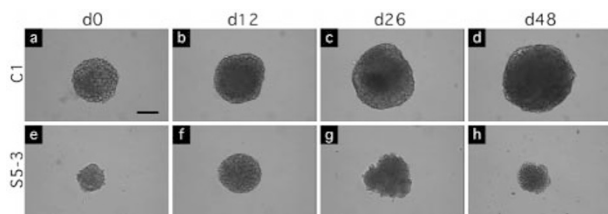


Figure 8 HSP25 overexpressing clones formed smaller and looser aggregates than C1. Phase contrast micrographs of aggregates of C1 (a–d) and S5-3 (e–h) clones, fixed, at d0, d12, d26 and d48 of differentiation indicate the dramatically altered size and morphology of S5-3 aggregates. Bar: 100 μ m

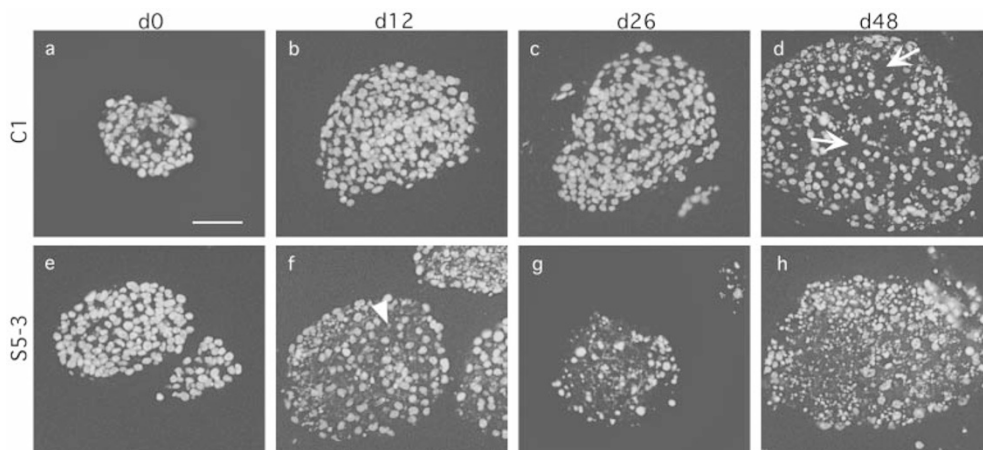


Figure 9 The nuclei degenerate during chondrocyte differentiation of the HSP25 overexpressing S5-3 clone. C1 (a–d) and S5-3 (e–h) aggregates were fixed and sections were labeled with Hoechst 33342 at d0, d12, d26 and d48 of chondrocyte differentiation. Arrows point to black areas corresponding to lacunae and extracellular matrix present in C1 aggregates at d48 (d). The arrowhead shows bright spots which presumably indicate the degenerating nuclei of dying cells (see text). These spots are visible in S5-3 aggregates from as early as d12 (f) and increase in quantity at d26 (g) and d48 (h). Bar: 100 μ m

dying, due to a process which could begin even earlier, between d12 and d26 of differentiation and which is not observed in C1 parental cells.

Reduced glutathione level in control and HSP25 overexpressing clones

Given that HSP25 has been proposed to protect against apoptosis via the metabolism of glutathione,^{29,30} we were interested in following the level of reduced glutathione (GSH) in both clones, throughout the differentiation process (Figure 10). At d0, the level of GSH is 2.5 higher in S5-3 than in C1 aggregates. A significant difference was also detected in non-differentiating dividing cells, between the three clones C1, S4-2 and S5-3 : GSH concentration was approximately 7.6, 22.5 and 16.6 nmoles/mg total protein for C1, S4-2 and S5-3 respectively (data not shown). These data suggest that

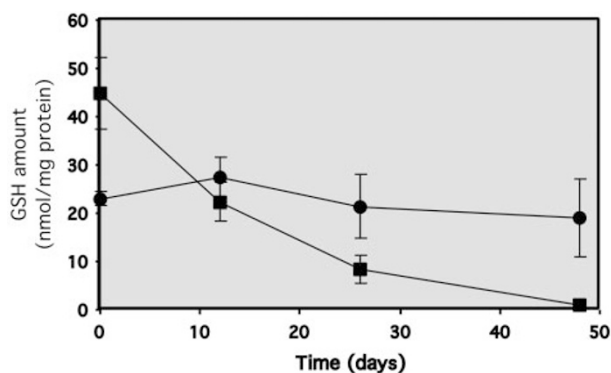


Figure 10 The level of reduced glutathione is altered during chondrocyte differentiation of the C1 and S5-3 clones. Reduced glutathione (GSH) was measured in triplicate for each clone and at each time point over 48 days of differentiation and normalized to total protein content. Although S5-3 cells initially contained significantly higher levels of GSH, this level dramatically decreased during differentiation, while the content of GSH in C1 cells remained constant. ■ S5-3 ● C1 aggregates

the higher level of GSH is related to the overexpression of HSP25. After d0, the level of GSH remains constant in C1 cells, at around 20 nmoles/mg protein. In S5-3 aggregates, this level, initially higher than in C1, rapidly falls from 45 nmoles/mg protein (d0) to 20 nmoles/mg protein (d12) and then to less than 10 nmoles/mg protein at d26, being undetectable after d48. These variations seem to be correlated to the dissociation of the aggregates and the changes in the appearance of the nuclei described above.

Discussion

Though HSPs have long been studied with regard to their role in cell protection in response to various stresses, recent studies give insights into their possible specific roles in development. HSP25 is the paradigm of small heat shock proteins in mammalian cells. It has been studied in a few cellular systems and to a lesser extent in the whole animal. Its study in postnatal or adult mouse or rat, as well as in the developing embryo^{3,4,9,10,39} underlines its specific and very high expression in several organs and structures.

A few cellular systems only have been used to study HSP25 role in cell differentiation.^{27,28,40-43} Recently, P19 cells have been analyzed more extensively.⁸ HSP25 content increases drastically during cardiomyocyte differentiation and fewer beating cells can be seen when HSP25 expression was inhibited by antisense transfection. But HSP25 does not seem to be necessary for neuronal differentiation of P19 cells.

We were interested in investigating HSP25 expression and its role in another differentiation process : chondrogenesis. HSP25 content has been shown to increase throughout chondrogenesis of the mouse embryonic limb.⁹ The amount of this protein is far lower than in striated muscles (cardiac and skeletal), but it increases in parallel with chondrocyte growth, maturation and with hypertrophic chondrocyte formation when extracellular matrix calcifies. C1 cells are a good cellular system to study mouse chondrogenesis *in vitro*.³³

Overexpression of HSP25

Stable overexpression of HSP25 was used in the C1 cellular system to understand the small HSP activity in this differentiation process. Overexpression of genes has proven to be very useful in cell systems where redundant genes exist.⁴⁴ In contrast to antisense-type experiments, this method can lead to enhanced phenotypes which are not hidden by the contribution from redundant genes. In the case of HSP25, α B-crystallin might play a similar role, as well as, though their similarities are lower, MKBP,³⁶ p20,⁴⁵ HspB3⁴⁶ or the recently discovered cvHsp.⁴⁷ Thus, we transfected C1 cells with the complete HSP25 cDNA and selected stable transformants. Two overexpressing clones were obtained, showing different amounts of HSP25. These cells did not show any difference in the content of the other HSPs investigated (HSP84, α B-crystallin and MKBP).

HSP25 and the cytoskeleton

Overexpression of HSP25 is accompanied by morphological changes. C1 cells are polygonal cells, about 40 μ m in diameter. S4-2 were dramatically modified in their shape, being long and thin, with thin extensions, in a fibroblast-like shape. S5-3 cells are also morphologically distinguishable from C1 cells, although those alterations are less evident than for S4-2.

Overexpression of HSP25 was also associated with a reduction in cell growth rate. The doubling time was 157% that of C1 in S4-2 cells and 135% in S5-3. Thus, overexpression of HSP25 tends to reduce cell proliferation in a way which is directly correlated with the small heat shock protein amount. Cell growth rate alteration due to HSP25 underexpression has been described : the cell division rate seems to be reduced in cells where HSP25 content is lowered (Arrigo, personal communication,²⁴) but it has not been quantified. In cellular systems which overexpress HSP25, the cell growth rate has not been extensively investigated.^{12,48-50} Reduced expression as well as overexpression of HSP25 could lead to growth rate reduction, which emphasizes the importance of defined quantities of HSP25 in the cells to allow proper division. How a modification in HSP25 cellular amount could modify the cell growth rate remains unanswered.

HSP25 has been described *in vitro* as interfering with the actin filaments, preventing polymerization of globular actin into filaments, through reduction of the capping activity.¹⁵ The modifications of cell morphology and growth rate in clones which overexpress HSP25 and the correlation of these effects with the level of overexpression suggest that HSP25 could interfere with the cytoskeleton. However, no modification was detected in the actin amount and its distribution in the cells. This does not exclude local modification in the cytoplasm of the cells, but it was not detected by immunofluorescence cytology.

The role of HSP25 could be dependent on its phosphorylation state.^{14,51} The polymerization state of this protein also contributes to the regulation of its activity.⁵² In response to stress, HSP25 aggregates of higher molecular weight (up to 700 kDa) form, which are active, whereas smaller aggregates (<200 kDa), inactive, are present in unstressed cells. Further investigations would have to be conducted on S4-2 and S5-3 clones to analyze the phosphorylation and polymerization states of HSP25, in exponentially growing as well as aggregating cells.

HSP25 and chondrocyte differentiation

As HSP25 expression has been shown to increase throughout differentiation of the mouse embryonic limb,⁹ we were interested in evaluating the role of HSP25 overexpression in chondrogenesis *in vitro*. The effect of HSP25 overexpression was followed only in C1 and S5-3 clones, since early dissociation of S4-2 aggregates did not allow us to collect enough aggregates of this clone to study chondrogenesis (for discussion of the role of HSP25 in disaggregation, see below). S5-3 differentiation seems to be altered. There is no formation of cartilage or lacunae such as can be seen in C1

aggregates. This was confirmed by collagen I and II studies (Figure 7). Whereas collagen II, a specific marker for cartilage formation, increases until d26 in C1 aggregates, it is present at d0 in S5-3 cells but totally absent from d12. This corresponds to a clear modification in collagen II expression. Similarly, collagen I, a marker of chondroprogenitors, is not expressed in S5-3 aggregates during differentiation except at d0, when it is transiently expressed during the differentiation of C1 cells, with a peak at d12. Thus, HSP25 overexpression leads to dramatic changes in the synthesis of the specific markers of chondrogenesis.

Cells within the S5-3 aggregates appear to have more diffuse borders and less defined nuclear envelopes than C1 cells (Figure 7B). Nuclei staining by Hoechst was completed to know whether cell death was occurring (Figure 9). C1 aggregates displayed intact nuclei at all stages of differentiation. In contrast, nuclear integrity was strongly altered in S5-3 cells as early as d12, even before the modifications which could be observed in the morphology of the aggregates. Only a few nuclei could be seen at d26 or d48. Clearly, most of the cells died in the aggregates as soon as d26 or even earlier. Whether this death is due to apoptosis or another mechanism remains to be studied.

It has been shown in a few cellular systems that reduced expression of HSP25²⁴⁻²⁶ may induce a change in cell differentiation. The group of Arrigo has proposed that HSP25 could play a role as a switch between cell differentiation and apoptosis.^{27,28} In ES cells or olfactory neurons undergoing differentiation, a transient expression of HSP25 accompanies arrest of cell proliferation and entry into differentiation. When this increase is altered, more cells undergo apoptosis. This protection against apoptosis may be related to redox metabolism through glutathione regulation.^{30,53} Thus, HSP25 could protect cells against apoptosis both under stressful conditions⁵⁴⁻⁵⁶ and during differentiation. Preville *et al.*²⁹ have shown that HSP25 can increase reduced glutathione regeneration through control of the glucose-6-phosphate dehydrogenase activity.

Therefore, reduced glutathione content was followed throughout differentiation of both C1 and S5-3 cells. Whereas its level remains constant in C1 cells, it is initially higher in S5-3 cells and then dramatically decreases. The initially high level of reduced glutathione in S5-3 clone may be related to the overexpression of HSP25. In non-differentiating dividing cells, reduced glutathione content was also higher in S5-3 and S4-2 clones than in C1 : it was approximately 7.6, 22.5 and 16.6 nmoles/mg total protein for C1, S4-2 and S5-3 respectively. Once again, these differences are correlated with the level of expression of HSP25. However, despite the high level of HSP25 protein in clone S5-3, a high level of reduced glutathione was not maintained throughout the whole process of chondrocyte differentiation treatment and cell death was not prevented.

HSP25 and adhesion molecules

The smaller size of the cellular aggregates formed from HSP25 overexpressing clones and their dissociation during

chondrogenesis (between d0 and d12 for S4-2 and more slowly for S5-3) suggest that this protein could be involved in adhesion properties of these cells. Chondrogenesis is known to be highly dependent on the first step of condensation, both *in vivo* and *in vitro*. This differentiation process requires condensation of mesenchymal cells prior to proliferation, maturation and differentiation of chondrocytes. N-cadherin has been shown to be essential for this initial step in chicken chondrogenesis.^{57,58} It enables cell-cell interaction, in connection with fibronectin, laminin and $\alpha 5\beta 1$ integrin receptor,⁵⁹ thus enabling aggregate assembly. N-cadherin is highly expressed in the early stages of cellular condensation and diminishes throughout differentiation. N-CAM has been described as being secondarily expressed.⁵⁸ It contributes to stabilization of the cell-cell interactions initiated by N-cadherin expression. The expression profile of this adhesion molecule is similar to that of N-cadherin but slightly delayed. More recently, Chimal-Monroy *et al.*⁶⁰ were also able to detect N-cadherin and N-CAM proteins through Western-blot analyses of cell culture from forelimb buds of mouse.

The altered morphology observed in the aggregates which tend to dissociate during differentiation (see Figure 8), at a rate which correlates with HSP25 overexpression level, supports the hypothesis that HSP25 overexpression could interfere with adhesion molecules in a way which reduces cell condensation. Adhesion molecules such as N-cadherin, N-CAM and integrins, which are involved in the early stages of chondrogenesis, are also known to interact with microfilaments and intermediate filaments of the cytoskeleton, either directly or through intracytoplasmic proteins.⁶¹ As HSP25 is known to interact with actin and intermediate filaments, this protein could play a role as a linking protein between integrin receptors and the cytoskeleton.

In summary, the overexpression of HSP25 in C1 cells leads to dramatic changes in the shape of the cellular aggregates, possibly by interfering directly or indirectly with cell adhesion molecules, and thereby prevents chondrogenic differentiation.

This phenomenon appears to be important since HSP25 expression has also been reported to be increased in several tumor cells which are resistant to chemotherapeutic agents;^{37,50} for a review, see.⁶² If overexpression of HSP25, as we show here, also reduces or modifies cell-cell adhesion properties, this could be of major clinical consequences since the HSP25 overexpressing tumor cells, which appear to be resistant to cytotoxic drugs, could also more easily separate from the tissue and promote metastases.

Materials and Methods

Transfection

C1 cells were transfected by CaPO₄ precipitation using the plasmid pKJ23 encoding hygromycin resistance alone or with a 10-fold excess of the expression vector pSVK3 (Pharmacia, Sweden) engineered to contain the murine HSP25 cDNA in sense orientation under the control of the SV40 promoter.^{27,63} Resistant cells were selected in 200 μ g/ml

hygromycin and single clones isolated and tested for the level of HSP25 protein by immunoblot. Among 17 independent clones analyzed, two showed a significant and reproducible increased level of HSP25 on immunoblots.

Cell culture and differentiation

C1 cells and transfectants were cultured in tissue-culture dishes (Falcon) at 37°C in 7.5% CO₂ water-saturated atmosphere in DMEM medium (Gibco) containing 10% heat-inactivated fetal calf serum, 1 mM pyruvate, 2 mM glutamine, 50 µg/ml streptomycin and 50 units/ml penicillin. To carry out the differentiation, C1 or transfectants were seeded at 3 × 10⁵ cells per 10 ml of culture medium in untreated plastic dishes to form cell aggregates. After 9 days (day 0), the aggregates were re-fed with DMEM (serum-free) supplemented with 1 mM pyruvate, 2 mM glutamine, 50 µg/ml streptomycin and 50 units/ml penicillin and induced to differentiate along the chondrogenic pathway by addition of 10⁻⁶ M dexamethasone (Sigma). At day 40, 50 µg/ml ascorbic acid (Sigma) and 1 µM thyroid hormone T3 (Sigma) were added to the differentiation medium.

Cell growth kinetics

C1 cells and transfectants were seeded at 10⁵ cells per 6 cm tissue-culture dish. Over the next 9 days, three separate dishes were harvested daily for each clone. Cells were removed by trypsin treatment, washed with PBS and counted with a hemocytometer. The number of cells at each point and for each clone was estimated by taking the mean of these three independent values. The different growth curves obtained were compared by Super-Anova statistic test.

Gel electrophoresis and immunoblotting

Protein extracts were prepared by washing cells in 4°C PBS, scraping into Laemmli sample buffer with 10 mM dithiothreitol and then heating for 10 min at 95°C.⁶⁴ Aliquots were assayed for protein concentration using the Bio-rad protein assay kit (Bio-rad) and equal amounts of protein were loaded on an SDS-15% polyacrylamide gel. Prestained low-range SDS-PAGE standards from Bio-Rad were used. As an internal control on HSP25 immunoblots, 100 ng of purified HSP25 (SPP-710, StressGen, Canada) was run with the standards.

Proteins were electrotransferred onto Hybond-ECL nitrocellulose (Amersham). Equal loading of protein was verified by staining the membrane with Ponceau red. Immunoblots were performed as described in the ECL kit (Amersham Corp.). The general dilutions used were 1:5000 for first antibodies (anti-HSP25 SPA-800 from StressGen, anti-HSP84 PA3-012 from Affinity Bioreagent) and 1:15000 for peroxidase-conjugated secondary antibodies (anti-rabbit W4011 from Promega).

Immunocytochemistry

Cells for immunocytochemistry were grown on gelatin-coated sterile glass coverslips. At 50% confluence, cells were washed with 4°C PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Coverslips were washed in PBS and stored at 4°C in PBS. Immunocytochemistry of coverslips was performed in a humid chamber. Coverslips were incubated in 0.1% Triton X in PBS for 5 min to permeabilize the cells. Coverslips were blocked for 1 h in 3% BSA in PBS, then incubated with primary antibody (1/100 in 3% BSA/PBS) for 1 h: anti HSP25 (SPA801 from StressGen), anti

HSP84 (PA3-012 from Affinity Bioreagents), anti αB-crystallin (NCL6ABcrys from Novocastra-Tebu) or anti MKBP (gift from A Suzuki). The coverslips were washed three times in PBS and once in 3% BSA/PBS (5 min), then incubated with 1/400 anti rabbit IgG-Cy3 (111-165-003 from Interchim), 10 µg/ml Hoechst 33342 (Sigma) and possibly 1 µg/ml FITC-phalloidin (Sigma), 30 min in the dark. After washing four times in PBS, coverslips were mounted on slides with Mowiol, dried overnight, and examined on a Leica DMRB microscope. Photographs were taken with KODAK 1600 film, and these were scanned and images assembled using Photoshop and Canvas.

Image analysis of aggregate size distribution

Images of aggregates were analyzed with NIH-Image, in order to estimate the projection surface of the aggregates. From the projection surface of the aggregates, that more or less correspond to spherical masses, we deduced an estimated radius of the aggregates.

Aggregate sections and immunohistochemistry

For immunohistochemical analyses of cell aggregates, aggregates were collected, rinsed twice in PBS and fixed in 4% paraformaldehyde in PBS, 2 h at room temperature. They were embedded in 4% low melting point agarose (Appligen), prior to inclusion in Parasplast plus* (Sherwood Medical). Three µm sections cut on a Leica microtome were laid on treated slides (SuperFrost*/Plus) in 0.2% gelatin, dried at 37°C and stored at 4°C. Before immunocytochemistry, the slides were cleared of paraplast in two Histo-clear (National Diagnostics) baths of 15 min, then gradually rehydrated through an ethanol series and soaked in PBS for 5 min. Immunocytochemistry was performed in a humid chamber. Free aldehydes were saturated with 50 mM NH₄Cl in PBS for 30 min. Sections were submerged for 30 min in a blocking solution (3% BSA in PBS), containing 0.5% Triton X-100 for permeabilization, and 0.3% H₂O₂ as substrate to block endogenous peroxidase activity of the tissues. The slides were washed three times 10 min in 3% BSA, 0.1% Triton X in PBS, then incubated with 1/100 anti collagen II (from Rockland) in 3% BSA, 0.1% Triton X in PBS for 1 h. After washing three times in 3% BSA, 0.1% Triton X in PBS, slides were incubated with 1/100 anti rabbit IgG-peroxidase in 3% BSA, 0.1% Triton X in PBS for 30 min. Peroxidase activity was revealed using 0.03% diaminobenzidine tetrahydrochloride (Sigma), 0.005% H₂O₂ in 0.1 M Tris-HCl (pH 7.6). The reaction was stopped in distilled water and the sections mounted in Mowiol. Observations were made on a Leica DMRB microscope. Photographs were taken with Kodak 64T film, scanned and images assembled as previously.

RNA extraction and Northern-blot analyses

Total RNA was extracted from cells and aggregates using the RNA-plus kit from Quantum Bioprobe. Northern-blots were prepared with 20 µg of RNA resolved on 1% agarose gels and transferred to Hybond-N⁺ membranes (Amersham). Double stranded DNA probes were labeled by random-priming with the Megaprime kit (Amersham) using (α³²P)dATP, while oligonucleotides were labeled by (γ³²P)ATP using T4 polynucleotide kinase. Hybridizations were carried out at 65°C for at least 15 h for double stranded DNA probes, while for oligonucleotides the temperature was gradually decreased until 30°C over 15 h after 30 min at 65°C. Washes were performed at 40°C, first with 2 × SSC, 0.1% SDS (2 × 15 min), then with 0.2 × SSC, 0.1% SDS (2 × 15 min). The membrane was exposed to X-OMATTM film (Kodak).

Measurement of reduced glutathione (GSH) level

Cells and aggregates at different stages were harvested and rinsed with PBS. 90% of each sample was analyzed for glutathione content⁶⁵ and the remaining 10% was used to measure protein concentration.⁶⁶

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