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Aberrant Extracellular and Dendritic Cell (DC) Surface Expression of Heat Shock Protein (hsp)70 in the Rheumatoid Joint: Possible Mechanisms of hsp/DC-Mediated Cross-Priming

Carla A. Martin,*† Steven E. Carsons,‡ Robert Kowalewski,‡ David Bernstein,† Michael Valentino,‡ and Frances Santiago-Schwarz2*†

We describe, in rheumatoid arthritis (RA), abnormalities in the expression and distribution of heat shock protein (hsp) and dendritic cells (DCs) that are conducive to cross-priming and DC cross-talk. As detected by ELISA, inducible (i)hsp70 was dramatically increased in RA synovial fluid (RASF) vs normal human and RA sera and osteoarthritis and gout synovial fluid. Immunoblot analysis of fresh RASF cells revealed marked increases in ihsp70 and activation of its transcription factor heat shock factor-1, compared with fresh normal peripheral blood cells. Flow cytometry and microscopy demonstrated high levels of ihsp70 on the surface of RASF myeloid DCs (but not normal myeloid DCs) that occurred concurrently with hspRs (CD91/CD14). ihsp70 present in RASF exhibited chaperoning potential, as indicated by the capture of ihsp70 present in RASF on the surface of normal DCs. Binding was partially competitively inhibited by excess α2-macroglobulin, indicating that hspRs in addition to CD91 participate in the capture process. These data indicate that ihsp70 may chaperone autologous Ags into immature RA synovial DCs via hspRs, and that cross-talk between DCs expressing ihsp70/hspRs reflects a disease process in RA. The induction of surface ihsp70 on normal cells after sublethal heat stress and the release of ihsp70 from normal DCs after inflammatory stress also suggest that the pattern of ihsp70 expression in RASF occurs in response to sustained stress. The Journal of Immunology, 2003, 171: 5736–5742.

Heat shock proteins (hsp) are evolutionarily conserved, stress-inducible molecules that promote protein stability and transport (1). Recent studies have focused on the interplay between hsp and innate and acquired immunity. Emerging concepts in acquired immunity are that hsp released from damaged cells chaperone immunogens into APCs such as dendritic cells (DCs) via specific receptors, and that they facilitate movement along the intracellular pathways associated with Ag presentation (1–6). The ability of hsp to dramatically increase the immunogenicity of chaperoned tumor Ags has prompted their use in cancer trials using DC-based cross-priming strategies to elicit antitumor T cell responses (2). hsp may be released from damaged cells after stress, whereas in normal unstressed cells, they are distributed in various intracellular compartments and are not expressed on the cell surface (1). In a variety of tumors, abnormal cell surface expression of inducible (i)hsp70 may occur on viable cells, for instance, upon recovery from sublethal stress (7, 8). The increased interaction of such tumor cells bearing surface hsp70 with immature DCs bearing hspRs and subsequent DC-mediated T cell immunity toward the tumor cells substantiates that hsp are important mediators of adaptive immunity (8).

hsp are thought to be important in the establishment and maintenance of autoreactivity in autoimmune diseases (2, 9, 10). They may be directly immunogenic, share immunogenic epitopes with various pathogens implicated in autoimmunity, and may be structurally linked with MHC genes (2, 11, 12). Recent findings indicating a role for hsp70 as a cytokine that induces secondary proinflammatory cytokine (TNF-α, IL-6) production may also be relevant to the establishment of autoimmune diseases (13). In an evolving scenario supported by animal models of autoimmune diabetes and lupus erythematosus, cross-priming with self-Ags occurs when apoptotic/necrotic cell uptake is accompanied by danger signals such as TNF that promote DC activation and maturation (2, 14, 15). Thus, in the proper setting, self-Ags present in damaged cells may be chaperoned by hsp into immature DCs for representation by MHC molecules on mature DCs. In rheumatoid arthritis (RA), physical interactions between hsp70 and MHC class II shared epitopes suggest that hsp70 participates in the autoimmune response (9, 16). Although abnormally elevated hsp70 mRNA and hsp70 have been described in RA synovial tissue relative to osteoarthritis (OA) synovial tissue (9), the distribution of hsp70 in the RA synovial fluid (RASF) space (which contains the bulk of immature DCs present in the joint and which may be a source of immunogen (17)) is unknown. Also unknown are the potential interactions of hsp70 with immature DCs in RA.
Materials and Methods

Study population and biological samples

Twenty-seven patients with RA diagnosed according to the 1987 revised criteria of the American College of Rheumatology were studied. Six patients were male; mean age was 61 (range, 39–84). All patients were receiving treatment at the time of sample collection. Most (>80%) of the patients were receiving disease-modifying agents including gold, methotrexate, sulfasalazine, hydroxychloroquine, leflunomide, and azathioprine. A few were treated with steroids and nonsteroidal anti-inflammatory agents. None of the patients in this study group were receiving TNF antagonist therapy. Synovial fluid (SF) and peripheral blood (PB) were obtained as part of routine clinical care. Cell-free SF and serum were cleared of any precipitate by centrifugation (1500 g for 15 min at 4°C) and immediately stored at −80°C until use. Ten patients with OA, 5 patients with gout, and 10 normal healthy controls were also included. Mononuclear cells (MNC) were prepared from heparinized samples using density centrifugation and resuspended in complete medium consisting of RPMI 1640 medium (Life Technologies, Grand Island, NY) with 2 mM L-glutamine, 10 mM HEPES, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5% pooled normal human serum (NHS). All culture conditions were low in endotoxin, as previously described by us (18). The study was conducted according to institutional guidelines.

ELISA

ihsp70 content in biological fluids was measured using a commercial quantitative sandwich ELISA (StressGen, Victoria, British Columbia, Canada), with a sensitivity of 200 pg/ml. Experiments were performed exactly as recommended. Results were detected using a microplate ELISA reader at 450 nm and are expressed as nanograms of ihsp70 per milliliter. The Abs used in the ELISA are specific for ihsp70 and do not cross-react with constitutive hsp70. Detection of ihsp70 was influenced by rheumatoid factor, as revealed in spike recovery and isotype Ab control studies.

Immunofluorescence (IF) studies

For ihsp70 cell surface staining, cells were resuspended in staining buffer (PBS/0.1% sodium azide/1% albumin) and incubated for 30 min on ice with a monoclonal anti-ihsp70 Ab that is specific for ihsp70 and that does not cross-react with constitutive hsp70 (StressGen) or isotype-matched control Abs, incubated with secondary Ab linked to Alexa Fluor 488 (Molecular Probes, Eugene, OR) for another 30 min, fixed in 1% PBS-buffered formalin, and then analyzed by flow cytometry (FACSsort, BD Biosciences, Mountain View, CA). Where indicated, an Ab detecting both the inducible and constitutive forms of hsp70 (Sigma-Aldrich, St. Louis, MO) was also used to stain cells. The flow cytometer was calibrated using Calibrite beads (BD Biosciences), and 10,000 events were acquired. Results were obtained using CellQuest analysis software (BD Biosciences) and are expressed as percentage of positive cells after subtracting negative control values. Microscopic visualization of surface-stained cells was performed after depositing stained cells onto slides by cytocentrifugation and counterstaining with Reissert’s fluid (50 μg/ml) and for multiple labeling of cells, Abs or isotype controls directly linked to either PE or FITC were used. Abs to DR and CD86 were obtained from BD Biosciences; anti-CD91 was obtained from Dako (Glostrup, Denmark). For total cell staining (surface and intracellular) and mAbs to DR and Perry, Gaithersburg, MD) and anti-rabbit IgG (Amersham, Piscataway, NJ), respectively, linked to HRP. Immunoreactivity was revealed by chemiluminescence using ECL reagent (Amersham). Densitometric analysis was performed using a ChemiImager 4400 (Alpha Innotech, San Leandro, CA).

Statistical analysis

Student’s t test and Mann-Whitney rank tests were performed using statistical software (SigmaStat; Jandel Scientific, San Rafael, CA). A value of p < 0.05 was considered to be a significant difference.

Results

Distribution of soluble ihsp70

Levels of ihsp70 were increased >90-fold in RASF compared with normal human sera (367 vs 4 ng/ml; p < 0.0001) (Fig. 1). Compared with RA sera, RASF was increased ~30-fold (p < 0.0001) (Fig. 1). Although trends toward increases (3-fold) in ihsp70 were noted in RA sera vs normal sera, results were not significant (p = 0.064). OA and gout SF contained higher ihsp70 levels than normal sera (24 and 59 vs 4 ng/ml, respectively; p = 0.02). However, relative to RASF, ihsp70 levels were reduced in OA and gout SF (p ≤ 0.0025) (Fig. 1). Comparisons of paired SF and serum samples in a subset of RA patients (n = 6) confirmed marked increases in ihsp70 in RASF (46- to 142-fold for RASF vs RA sera; p = 0.01; Table I). None of the RA sera tested (n = 10) exhibited soluble ihsp70 levels that were increased above RASF. Detection of a ~70-kDa protein by immunoblot analysis using anti-ihsp70 Ab (StressGen) confirmed reactivity in the ELISA as ihsp70 (Fig. 1C).

Immunoblot analysis of ihsp70 in RASF cells

Synovial lining fibroblasts in RA have been previously shown to produce large amounts of ihsp70 (9). Thus, the excess of ihsp70 in RASF may be derived from fibroblasts that are in close proximity to SF. Alternatively (or additionally), hemopoietic cells present in RASF may be a major source of ihsp70. In support of the latter, levels of ihsp70 in fresh RASF cells (0 h) were ~4-fold greater than levels present in fresh normal cells and were comparable to the levels achieved in normal cells 48 h after heat stress (Fig. 2).

FIGURE 1. RASF contains greatly elevated levels of ihsp70. A and B, As detected by ELISA, ihsp70 levels were dramatically increased in RASF compared with normal and RA sera and with OA and gout SF. For RASF, n = 20; for RA and normal sera (NL SE), n = 10; for OA SF, n = 10, and for gout SF, n = 5. For RA SF vs RA sera, p < 0.0001; for RA SF vs OA and gout SF, p < 0.0025; and for OA SF vs gout SF, p < 0.025. C Detection of a ~70-kDa protein by immunoblot analysis using anti-ihsp70 Ab (StressGen) confirmed reactivity in the ELISA as ihsp70.
Immunoblot analysis of OA SF (OASF) cells did not reveal increased levels of ihsp70 over RASF (data not shown).

Surface distribution of ihsp70 on RASF cells

Microscopy and flow cytometry IF analysis of freshly prepared normal MNC and RASF cells revealed striking differences in the cellular distribution of ihsp70 (Fig. 3). Although immunoblot analysis showed that fresh RASF cells contained far more ihsp70 than fresh normal cells (Fig. 2), both flow cytometry and microscopy revealed that most of the ihsp70 was present in myeloid cells (Fig. 3). As determined by flow cytometry, 3.3 ± 1.0% of normal cells vs 42.5 ± 13.5% of RASF cells expressed surface ihsp70 (p = 0.024, for normal vs RA). Most of the surface ihsp70 was on mononuclear myeloid cells; polymorphonuclear cells that were present in RASF demonstrated much weaker cell surface reactivity (although they contained intracellular ihsp70). IF studies performed with an Ab that detects both the constitutively expressed and inducible forms of ihsp70 (Sigma-Aldrich) also revealed a lack of surface expression on normal cells (data not shown).

Coincident expression of ihsp70 and hsp70Rs on immature DCs

Multiple label IF analysis demonstrated that surface ihsp70 on freshly isolated RASF myeloid cells occurred on DC precursors, and that hspRs were coexpressed with surface ihsp70 (Fig. 4A). As previously noted by us (17), RASF contained CD86+/DR+ DCs and CD14+ DC precursors. CD91, the universal hspR (4) was highly coexpressed with CD14 (which can also bind hsp70 via the CD14 surface complex) (13, 21). The majority of the CD14+ and CD91+ DC precursors coexpressed surface ihsp70, whereas only a subset of ihsp70+ cells lacked CD14 and CD91. Freshly isolated normal PB DC precursors exhibited CD91 and CD14, but did not express surface ihsp70 (Fig. 4B, left panels). Surface expression of ihsp70 was induced on normal CD14+ and CD91+ DC precursors shortly (4–6 h) after heat stress, albeit at much lower levels than fresh RASF cells (Fig. 4B, right panels).

ihsp70 present in RASF is captured by normal DC precursors

To determine whether ihsp70 present in RASF could physically interact with DC precursors, we incubated freshly isolated normal PB MNC (NLPBMNC) with RASF and then performed IF with anti-ihsp70 Ab. As expected, only low levels of surface ihsp70 were detected on normal cells before treatment with RASF. After incubation with RASF, marked increases in the number of CD91+ positive myeloid cells expressing surface ihsp70 occurred (Fig. 5, upper panels) (p = 0.029 for normal cells incubated with RASF vs untreated cells; n = 4). These increases were similar to those obtained when normal cells were incubated with recombinant human ihsp70 (StressGen) (Fig. 5, lower panels). The physical association of ihsp70 contained in RASF or recombinant ihsp70 with

| Patient | ihsp70 RASF (ng/ml) | ihsp70 RASE (ng/ml) | Ratio (SF:SE) |
|---------|---------------------|---------------------|--------------|
| 1       | 106.5               | 0.78                | 136          |
| 2       | 405                 | 2.85                | 142          |
| 3       | 97.2                | 0.78                | 125          |
| 4       | 681                 | 14.8                | 46           |
| 5       | 592                 | 9.34                | 63           |
| 6       | 523                 | 7.05                | 74           |

*Paired SF and serum (SE) were obtained from RA patients and assayed by an ihsp70-specific ELISA, as outlined in the text; p = 0.01 for RASF vs RASE.

FIGURE 2. Increased expression of ihsp70 in RASF cells. Protein (10 µg) obtained from total cell lysates of unstressed (0-h) and post-heat-stressed (1- and 48-h) NLPBMNC and freshly isolated RASF cells was separated by SDS-PAGE, transferred to nitrocellulose membranes, and then probed with anti-ihsp70 Ab. RASF cells exhibited a 4.1-fold increase in ihsp70 compared with unstressed NLPBMNC (baseline equals 1-fold expression).

FIGURE 3. Abnormal distribution of ihsp70 on RASF cells. Total (intracellular and surface) and surface ihsp70 was detected in freshly isolated NLPBMNC (A) and RASF cells (B) by flow cytometry and microscopy. For microscopy, IF-stained cells were deposited onto slides and then counterstained with propidium iodide. Arrows designate the corresponding dot plot analysis of stained cells. Lymphoid and myeloid cells were differentiated by forward and side-angle light scatter and by morphology. A representative experiment is shown (n = 3 and 6 for RA and normal (NL), respectively). For NL, surface ihsp70, 3.3%; for RA, surface ihsp70, 42.5%. Original magnification, ×40. Control represents isotype Ab control.

FIGURE 4. Coincident expression of ihsp70 and hsp70Rs on immature DCs present in RASF cells. A, Freshly isolated RASF cells stained for surface ihsp70, hspRs (CD91, CD14), DR, and CD86. B, Unstressed (left panels) and post-heat-stressed NLPBMNCs stained for surface ihsp70 vs CD14 and CD91. Myeloid cells were gated based on scatter properties, and quadrants were set according to isotype controls. A representative experiment for each group is shown; n = 2 for NLPBMNC and RASF cells.
CD91 on DC precursors was suggested by the coincident increase in cells coexpressing CD91 and ihsp70 and is in agreement with prior studies that establish CD91 on DC precursors as a receptor for hsp70 (4). Surface binding of ihsp70 was limited to myeloid cells; normal lymphocytes incubated with RASF did not express reactivity with anti-ihsp70 Ab. Studies performed with cells that were fixed, incubated with RASF, and then stained with anti-ihsp70 yielded similar cell surface reactivity as cells that were metabolically inhibited (stained at 4°C in the presence of sodium azide) (data not shown).

**Partial inhibition of hsp70 binding**

Competitive inhibition with α2M (the initially described ligand for CD91) (22) was used to further study the physical association of ihsp70 present in RASF with surface CD91. As shown in Fig. 6A, freshly isolated normal PB myeloid cells expressed low levels of surface α2M. The ability of CD91 present on these cells to bind α2M was demonstrated by surface expression of α2M on the majority of myeloid cells after incubation with excess α2M. CD91 binding sites were saturated, as noted by the similar percentages of

**FIGURE 5.** ihsp70 present in RASF is captured by normal DC precursors. Freshly isolated NLPBMNC were incubated with RASF (upper right panel), buffer solution (PBA) (left panels), or as a positive control, recombinant ihsp70 (200 ng) (lower right panel) for 30 min on ice. Cells were then extensively washed and analyzed by flow cytometry using anti-ihsp70 and -CD91 (or isotype controls). The myeloid population was gated based on scatter properties, and quadrants were set according to isotype controls. Results represent percentage of cells coexpressing ihsp70 and CD91, after subtracting control values. A representative experiment using RASF containing >200 ng/ml ihsp70 is shown; n = 4 for RASF, and n = 2 for recombinant ihsp70.

**FIGURE 6.** Partial inhibition of ihsp70 binding by α2M on myeloid cells. A. CD91 binding to α2M (the originally described ligand for CD91) was indicated by increases in surface α2M expression after incubation of freshly prepared untreated NLPBMNC with either activated α2M or RASF (known to contain activated α2M), performed as described in the text. One of two similar experiments is shown. Lines delineating positivity were set based on results obtained with isotype control Ab. B. Preincubation of myeloid cells with excess α2M results in partial inhibition of ihsp70 binding (mean reduction, ~60%; n = 2). Left panel represents freshly isolated NLPBMNC that were placed in PBA buffer (Untreated) and stained with anti-ihsp70 Ab; the middle panel represents cells incubated with RASF and then stained with anti-ihsp70 Ab; the right panel represents cells pretreated with activated α2M followed by RASF and then stained with anti-ihsp70 Ab. Quadrants were set according to isotype controls. For A and B, myeloid cells were gated according to light scatter properties, and percentage indicates numbers obtained after subtracting control values.

**FIGURE 7.** Nuclear translocation/activation of HSF-1 in RASF cells. A. Fresh NLPBMNC and RASF cells were deposited onto slides, permeabilized, and stained by indirect IF for the hsp70 transcription factor HSF-1. The left panel represents staining with isotype control; all samples were counterstained with propidium iodide. Note the predominant nuclear staining in RASF cells and the cytosolic-restricted staining in normal cells. The inset represents nuclear staining in NLPBMNC 1 h post-heat-stress stained with anti-HSF-1. Original magnification, ×40. B. Immunoblot analysis of HSF-1 in unstressed (0 h) and post-heat-stressed (1 and 24 h) NLPBMNC and fresh RASF cells. The active form of HSF-1 is represented by a higher molecular mass. For each group in A and B, n = 2. A dotted line is drawn to depict shift in molecular mass.

**FIGURE 8.** Stress-induced release of ihsp70. NLPBMNCs were subjected to nonlethal heat stress (HS) (42°C for 45 min) in complete medium or to inflammatory stress (medium containing 5% RASF) or left untreated in complete medium (NHS). Cell-free supernatants were then collected at various time points and analyzed for ihsp70 by ELISA. To discriminate between the amount of ihsp70 released from RASF-treated NLPBMNCs and ihsp70 originally contained in the cell-free RASF, values obtained for medium containing 5% RASF alone were subtracted from the values obtained for supernatants collected from RASF-treated NLPBMNCs. The data represent the mean ± SEM; n = 3–4 for each group.
α₂M- and CD91-positive cells (data not shown). Interestingly, incubation with RASF, which has previously been shown to contain increased levels of modified α₂M (23, 24), also yielded a net increase in surface α₂M expression (although fewer cells were positive). Preincubation of PB MNC with excess α₂M followed by RASF resulted in partial inhibition (~60%) of ishsp70 binding to myeloid cells as shown in Fig. 6B. Thus, although CD91 may contribute to capturing ishsp70 present in RASF, other receptor interactions are also likely involved.

Activation and nuclear translocation of HSF-1

As further evidence for the increased synthesis of ishsp70 in RASF myeloid cells, we investigated whether the hsp transcription factor HSF-1 was abnormally activated in RASF cells. As shown in Fig. 7A, center panel, the distribution of HSF-1 detected by immunocytochemistry in unstressed NLPBMNC was restricted to the cytoplasm. As reported by others (9), this pattern represents inactive HSF-1 and is consistent with the lower levels of ishsp70 in unstressed cells. In marked contrast, fresh RASF cells, especially cells displaying myeloid and DC features, exhibited intense nuclear staining (Fig. 7A, right panel), which is consistent with active transcription of ishsp70 in RASF cells and thus increased expression. Nuclear staining for HSF-1 in RASF lymphocytes was much less pronounced. Normal myeloid PB cells (including monocyte DC precursors) exhibited nuclear staining 1 h after heat stress (Fig. 7A, inset). Immunoblot analysis with anti-HSF-1 Ab revealed a lower molecular mass band representing inactive HSF-1 in unstressed normal cells and a higher molecular mass band representing active hyperphosphorylated HSF-1 in heat-stressed normal cells and fresh RASF cells (Fig. 7B).

RASF induces ishsp70 release

To test the intriguing possibility that ishsp70 can be released from viable cells under conditions of sublethal stress (13, 21), we exposed NLPBMNC (1 × 10⁶ cells/ml) to heat stress (42°C for 45 min) or to inflammatory stress (RASF) and then collected cell-free supernatants at various time points during culture at 37°C. Fig. 8 represents results obtained from ishsp70 ELISAs performed with these samples. As expected, supernatants obtained from unstressed cells (no heat) cultured from 0–1 h in NHS yielded low levels (<1 ng/ml) of ishsp70. After 48 h, some increases in ishsp70 occurred in these unstressed samples, indicating that ex vivo manipulation alone instigates a modest amount of ishsp70 release from cells (p = 0.0001, 0–1 vs 48 h). Higher levels were noted in NHS samples obtained 48 h after heat stress (p = 0.045 for 48-h post-heat stress vs 48-h unstressed culture). Supernatants obtained from cell cultures in medium containing RASF for 48 h exhibited ishsp70 levels that were increased above all other samples tested. In these samples, ishsp70 levels were 42-fold higher than 0- to 1-h samples, 3-fold higher than 48-h culture samples, and ~2-fold higher than 48-h post-heat stress samples (p = 0.043 for RASF vs 48-h heat stressed; p = 0.0014 for RASF vs 48-h culture). The mean number of apoptotic cells ranged from 0.87 to 2.3% in all of the cultures, as assessed by microscopic analysis of propidium iodide- and Wright-stained cells (>500 cells per slide). These low levels of apoptosis are in agreement with steady-state levels previously observed in normal cells (25).

Discussion

Growing evidence indicates that, depending on the environment, the acquisition of autologous cellular material by immature DCs can lead to either cross-priming or cross-tolerance (14, 26, 27). Presumably, in normal physiology, tolerizing DCs primed with self-Ags develop (in the absence of dangerous stimuli) to guard against autoimmunity. In autoimmune diseases, tolerance is overridden by the development of immunogenic DCs that respond to dangerous stimuli and that are capable of re-presenting acquired autoantigens to autoreactive T cells. In earlier work, we noted that abnormal expansion of monocyte (CD14)-derived DCs reflects a mechanism for generating inflammatory-type Th1 responses in RA, and that the RA joint environment (especially SF) provides an ideal setting for cross-priming events that favor autoreactivity (17). This scenario includes the presence of immature DCs with the potential to ingest autologous apoptotic cells and/or cellular debris, increased levels of numerous danger and DC growth signals (including TNF, GM-CSF, IL-1, IL-6, IL-13, etc.), and the potential for hsp release due to inflammatory or shear stress (9, 10, 17). In this study, we demonstrate particular abnormalities in the distribution and expression of ishsp70 and RASF DCs that, for the first time, link hsp/DC-mediated cross-priming to the RA disease process.

Ishsp70 was dramatically increased in RASF (cell-free) compared with OASF, gout SF, RA sera, and normal human sera (Fig. 1, Table I). Although increases in ishsp70 occurred in OASF and gout SF relative to RA sera and normal human sera, these increases were much less pronounced. Thus, while some increase in ishsp70 may be prompted by chronic inflammation in the OA and gouty joint, large elevations in ishsp70 may be a distinct pathogenic event in the autoimmune RA joint. In support of a specific role of hsp70 in RA pathology, hsp70 binds to certain amino acid sequences in the third hypervariable region of the MHC DRβ1 chain commonly found in the alleles carrying susceptibility to RA, but not other arthropathies (16). Immunoblot analysis of proteins obtained from RASF cells revealed marked up-regulation of ishsp70 in RASF cells compared with normal unstressed PB cells (Fig. 2). The increased expression of ishsp70 in RASF cells we report in this study, together with the previously described increases in ishsp70 in synovial lining fibroblasts (9), suggests that hsp may chaperone Ags acquired from cells of both hemopoietic and nonhemopoietic origin. In theory, uptake of such a wide variety of cells by immature DCs in a setting conducive to cross-priming could result in a wide repertoire of autoreactive responses and epitope spreading. Moreover, the promiscuity of hsp interactions with pathogenic and non-pathogenic molecules may help explain why so many Ags have been implicated as autoantigens in RA.

The predominance of activated transcription factor HSF-1 in DC precursors present in RASF (Fig. 7A) indicated that increased ishsp70 gene expression was responsible for increased ishsp70 protein levels. In light of recent data showing that STAT and HSF-1 cooperate to enhance ishsp70 gene expression and that STATs are induced in DCs by cytokines such as GM-CSF and IL-6 (28–30) that are present in excess in RASF, it is likely that STAT/HSF-1 interactions further amplify HSF1-mediated ishsp70 increases in the RA joint.

Microscopic and flow-cytometric analysis unexpectedly revealed substantial surface expression of ishsp70 on RASF cells (Fig. 3). Although many conditions, including inflammatory stress, are known to increase intracellular ishsp70 levels, reports of surface ishsp70 expression have been mostly limited to tumor cells (2, 8). In tumors, surface hsp has been linked to greater immunogenicity in vivo, perhaps due to the surface hsp acting as endogenous adjuvants that increase the synthesis of DC growth factors (2, 8). The surface distribution of ishsp70 on RASF cells was restricted to myeloid cells, especially DC precursors. In contrast to unstressed DC precursors obtained from normal PB that express CD91 and CD14 but lack surface ishsp70, RASF DC precursors coexpressed ishsp70 and hsp70Rs (CD14, CD91) (Fig. 4).
Several explanations exist for the surface expression of hsp70 on RASF DCs. Our results imply that hsp70 may be translocated to the surface from the cytosol, perhaps, in response to sustained stress, and/or that hsp70 is captured onto the DC surface from the extracellular space via hspRs. Given the high intracellular content of hsp70 in RASF immature DCs and the dynamic metabolic activity of these cells, we contemplate the idea that at least some surface hsp70 expression may be due to abnormal expression of intracellular processed hsp70 (11). The induction of surface hsp70 on normal DC precursors after sublethal heat stress (Fig. 4B) further implies that, in RA, surface hsp70 expression may occur in consequence to sustained stress. In support of the possibility that hsp70 is bound to the DC surface via hsp/hspR interactions and Ag chaperoning, we demonstrated that normal myeloid DC precursors capture hsp70 present in RASF (Fig. 5). Regardless of the mechanisms leading to surface expression, the detection of hsp70 on RASF DCs discloses protein-binding potential that may be of pathological significance. One attractive possibility is that cross-talk between DCs expressing surface hsp and DCs expressing hspRs reflects an Ag-independent DC activation process that results in the secretion of proinflammatory cytokines and DC growth factors. This scenario would be in agreement with the previously described role of hsp70 as a cytokine inducing proinflammatory cytokine production (13).

The participation of CD91 in capturing hsp70 present in RASF was indicated by the reduction of surface hsp70 binding after treatment with excess α2M (the originally described ligand for CD91; Fig. 6B). Although these results are in agreement with previous studies identifying CD91 as a receptor for hsp70, the partial inhibition we noted suggests that other receptors present on DCs such as CD14 and CD40 are also involved in capturing the hsp70 present in RASF. CD40 is more widely expressed on maturing DCs than DC precursors, and CD14 is expressed on DC precursors but is lost with DC maturation. Therefore, it seems likely that the state of DC maturation and the DC hspR profile contributes to the overall DC hsp binding potential (which is lost on fully mature DCs). In this regard, the wide distribution of DC maturation stages in RASF variously expressing CD14, CD91, and CD40 may reflect an environment especially conducive to cross-priming via hsp.

The cycle of inflammation involving hsp in RA may be perpetuated by stresses present locally in the affected joint. Inflammatory stress in the form of cell-free RASF prompted the release of hsp70 from viable NLPBMNCs during short-term culture (Fig. 8). The induction of hsp70 by RASF is consistent with the hsp70-promoting effects of danger molecules such as TNF in RASF (10). Thus, it is conceivable that, in the inflamed RA joint, inflammatory stress contributes to the high levels of extracellular hsp70 we measured in RASF. As previously noted by others for RA synovial tissue, shear stress may also contribute to the high levels of hsp70 in the joint (9). Because live DCs secrete small vesicles referred to as exosomes that are specifically enriched in hsp(s), including hsp70 (31–33), it is also possible that the hsp70 we measured originated from such vesicles. Consistent with the idea that hsp70 can be released from cells that are not frankly apoptotic or necrotic, we noted that the release of hsp70 from normal cells occurred in the context of low apoptotic events.

A growing consensus is that effective strategies for autoimmune diseases might include interference with DC-mediated cross-priming events that result in the activation of autoreactive T cells. We provide novel insight into how interactions between hsp70 and DCs might propagate disease activity in RA and a basis for further studies aimed at preventing the activation of autoreactive T cells. Moreover, our results showing that α2M present in RASF may be captured by CD91 (Fig. 6A) indicate that α2M, which displays the capacity to chaperone Ags into DCs for T cell cross-priming (22), also contributes to the RA cross-priming process. Future studies might include determining the effects of specific inhibitors of hsp70 such as deoxypergualmin (34) on hsp/DC-mediated cross-priming events in RA and other autoimmune diseases, assessing the interactions of other hsp family members, such as Ig H chain binding protein, and characterizing α2M/DC interactions in the RA joint.

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