The heat shock proteins (HSPs) are a family of intracellular proteins found in all eukaryotes and prokaryotes. Their functions are well characterized and are central to maintaining cellular homeostasis and in promoting cell survival in response to stressful cellular conditions. However, several studies provide evidence that specific members of the HSP family might be secreted via an unidentified exocytotic pathway. Here we show that exosomes, small membrane vesicles that are secreted by numerous cell types, contribute to the release of HSP70 from human peripheral blood mononuclear cells (PBMCs) in both basal and stress-induced (heat shock at 40 or 43 °C for 1 h) states. HSP70 release from PBMCs is independent of the common secretory pathway because Brefeldin A, an inhibitor of the classical protein transport pathway, did not block HSP70 release. Furthermore, we show that HSP70 release from PBMCs does not occur via a lipid raft-dependent pathway, because treatment with methyl-β-cyclodextrin, a raft-disrupting drug, had no affect on HSP70 release. To examine whether exosomes contributed to HSP70 release from PBMCs, exosomes were purified from PBMC cultures, and exosomal number and HSP70 content were determined. We demonstrate that although heat shock does not influence the exosomal secretory rate, the HSP70 content of exosomes isolated from heat shocked PBMCs is significantly higher than control. These data identify a novel secretory pathway by which HSP70 can be actively released from cells in both the basal and stress-induced state.

The concept that specific HSPs can be actively secreted was suggested 15 years ago following the demonstration that heat-shocked rat embryo cells rapidly released HSP70 and HSP110 (2). Importantly, several studies have confirmed these earlier findings, demonstrating that HSP70 is secreted from a variety of cell types in response to cellular stress (3–7). The HSP70 protein lacks a secretory signal sequence, and, consequently, cellular HSP70 release is unaffected by inhibitors of the common secretory pathway (2, 3). Although it has been suggested that cellular HSP70 release may be the result of non-specific processes, such as cell lysis, several lines of evidence argue against this notion (2, 3, 6). Interestingly, two recent studies have provided evidence that specialized membrane microdomains, termed lipid rafts, may play a role in HSP70 exocytosis (3, 5).

Given the critical intracellular roles played by HSPs it may appear counterintuitive that a secretory pathway exists allowing cells to release HSPs both basally and in response to cellular stress. However, a growing body of evidence suggests that specific members of the HSP family possess potent immunoregulatory functions (8–12). Recombinant human HSP70 stimulates the production of interleukin-1β, tumor necrosis factor-α, interleukin-6, and interleukin-12 and promotes the up-regulation of co-stimulatory (CD86) and antigen presenting major histocompatibility complex (MHC)II molecules in monocytes and dendritic cells (11, 13). Furthermore, cell surface-bound HSP70 renders tumor cells more sensitive to natural killer cell-mediated cytolytic attack (14, 15). These findings have led to the hypothesis that extracellular HSPs, released either as a result of cellular necrosis or via a stress-induced exocytotic pathway, may act as a potent danger signal to the immune system (9, 16).

Our initial experiments were aimed at determining whether heat shock at physiological/pathophysiological temperatures, i.e. during febrile states, stimulated the release of HSPs. Interestingly, in the course of conducting these studies, we were unable to confirm a role for lipid rafts in either heat shock-induced or basal HSP70 release from human peripheral blood mononuclear cells (PBMCs). Almost 10 years ago, Multhoff and Hightower (17) hypothesized that exosomes, 60–80-nm vesicles secreted following the fusion of multivesicular bodies (MVBs) with the plasma membrane, may provide a secretory pathway allowing cells to actively release specific HSPs. The concept of the exosome was initially formed following the observation that maturing reticulocytes “shed” transferrin recep-
Exosome-dependent Trafficking of HSP70

EXPERIMENTAL PROCEDURES

Reagents, Media, and Antibodies—Dulbecco's modified Eagle's medium (25 mm glucose), a minimal essential medium, RPMI 1640 cell culture medium, fetal bovine serum (FBS), penicillin-streptomycin, and phosphate-buffered saline (PBS) were from Invitrogen. Dexamethasone, isobutylmethylxanthine, acetamidophenol, 5,5′-dithiobis(2-nitrobenzoic acid), Brefeldin A (BFA), and methyl-β-cyclodextrin (MBC) were obtained from Sigma. The lactate dehydrogenase (LDH) viability assay kit was from Roche Applied Science. Cholesterol concentrations were determined using the Amplex Red cholesterol assay kit from Molecular Probes (Clayton, Victoria, Australia). Human insulin was obtained from Novonordisk (Bagvaer, DK). Bicinchoninic acid protein assay reagents were from Pierce Biotechnology.

The antibodies used were as follows: mouse anti-HSP70 monoclonal antibody (Stressgen Bioreagents, BC, Canada) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The HSP70 enzyme-linked immunosorbent assay kits were from Stressgen Bioreagents.

Cell Culture—PBMCs were isolated from freshly drawn peripheral venous blood obtained from healthy volunteers using the Ficoll-Paque separation technique. Briefly, whole blood was diluted in PBS and layered over 15 ml of Ficoll-Paque. Following centrifugation at 1400 × g for 30 min, the interphase (mononuclear cells) layer was collected and washed in PBS. Following centrifugation at 1200 × g for 10 min the supernatant was removed, and the cell pellet was resuspended and washed in PBS. Following a final centrifugation at 900 × g the supernatant was discarded, and cells were resuspended in 2–3 ml of cell culture medium (RPMI 1640 medium, 10% FBS, 1% penicillin-streptomycin). Cells were then counted on a hemocytometer and resuspended at 1 × 10^6 cells/ml before being added to either 6- or 24-well culture plates. L6 myoblasts and 3T3-L1 fibroblasts were grown to 70–80% confluence in 75 cm² flasks then seeded into 6-cm plates. Myoblasts were grown in α minimal essential medium supplemented with 10% fetal calf serum and differentiated into myotubes at 80% confluence in media containing 2% horse serum (3–4 days). Fibroblasts were grown in Dulbecco's modified Eagle's medium (25 mm glucose) with 10% fetal calf serum and differentiated into adipocytes 2 days postconfluence with the addition of 5% serum, insulin (0.5 mM), and isobutylmethylxanthine (25 mM) for 2 nights, followed by a further 2 nights in medium containing only serum and insulin, after which most cells had accumulated lipid droplets. Insulin was then removed for 45 h, upon which time cells were ready for experimentation.

Heat Shock—Cells were exposed to heat shock, either 40 or 43 °C, or control conditions (37 °C) for 60 min in humidified incubators. Cells were allowed to recover at 37 °C, 5% CO₂ for 4 h to allow for the synthesis and release of HSPs.

Exosome Isolation—Fetal bovine serum contains endogenous exosomes; therefore, prior to experiments in which exosomes were to be examined cell culture medium (RPMI 1640 medium, 10% FBS, 1% penicillin-streptomycin) was centrifuged overnight at 110,000 × g to remove endogenous exosomes. Exosomes were isolated according to the protocol of Savina et al. (20). Briefly, exosomes were collected from 6 ml of PBMC medium (6 × 10⁶ cells) cultured over 2–6 h. The culture media were centrifuged at 800 × g for 10 min to pellet the cells and then centrifuged at 12,000 × g for 30 min to pellet any cellular debris. Exosomes were separated from the supernatant by centrifugation at 110,000 × g for 15 h. The exosomal pellet was washed once in PBS and then resuspended in 200 µl of PBS (exosome fraction).

Exosome Quantification—To quantify the amount of exosomes released we assessed the activity of acetylcholinesterase, an enzyme that is specific to these vesicles (21). Acetylcholinesterase activity was assayed as described by Savina et al. (20). Briefly, 40 µl of the exosome fraction was suspended in 110 µl of PBS. 37.5 µl of this PBS-diluted exosome fraction was then added to individual wells on a 96-well flat bottomed plate. 1.25 µl of acetylcholinesterase and 0.005 µml of ethidium bromide (2-nitrobenzoic acid) were then added to exosome fractions in a final volume of 300 µl, and the change in absorbance at 412 nm was monitored every 5 min. The data presented represent acetylcholinesterase enzymatic activity after 30 min of incubation.

RESULTS

Temperature-induced Cellular Stress Increases Intra- and Extracellular HSP70 in Multiple Cell Types—To examine the cellular ubiquity of HSP70 release, we examined whether heat shock (40 or 43 °C for 1 h followed by recovery at 37 °C for 4 h) induced the active release of HSP70 compared with control conditions (37 °C for 5 h). As expected, heat shock (43 °C) resulted in a marked accumulation of intracellular HSP70 compared with control in L6 myotubes (Fig. 1A). Cells were exposed to BFA for the duration of the experiment (6 h). The efficacy of the BFA treatment in inhibiting protein secretion via the classical protein transport pathway was confirmed by analyzing the concentration of tumor necrosis factor-α and IFN-γ in supernatants obtained from PBMCs stimulated with either lipopolysaccharide, or phorbol 12-myristate 13-acetate and ionomycin.

Cholesterol Depletion—To deplete cellular cholesterol, cells (1 × 10⁶) were pretreated with MBC (5 or 10 mM) for 1 h at 37 °C. Cells were then washed, centrifuged for 10 min at 300 × g, and resuspended in MBC-free medium for the duration of the experiment. The cell pellets were collected, and the intracellular cholesterol concentrations were determined using a kit according to the manufacturer's instructions.

L6—LDH was determined on a microplate reader at 492 nm using a commercial kit as recommended by the manufacturer.

Statistics—Data are presented as means ± S.E. Comparisons between groups were performed using the paired samples Student's t test or repeated measures analysis of variance (RM ANOVA) where appropriate. Where a significant F ratio was found (RM ANOVA) differences between means were inspected with Tukey's Honestly Significant Difference post hoc test. A p value < 0.05 was considered statistically significant.
shock had any cytotoxic affects, the LDH concentration in the cell culture media was determined. Crucially, heat shock (40 or 43 °C) had no effect on LDH release (data not shown) compared with control (37 °C). In addition, we performed trypan blue exclusion analysis. Cell viability was always greater than 90%, and we did not observe any differences between heat shock (40 or 43 °C) and control (37 °C) conditions. These data demonstrate that heat shock induces the release of HSP70 from a variety of cell types and that this release is independent of cell death.

**HSP70 Release Is Unaffected by Inhibition of the Common Secretory Pathway or the Disruption of Lipid Rafts**—To examine the involvement of the common secretory pathway in HSP70 release PBMCs were pretreated for 1 h with BFA (a fungal metabolite that inhibits protein transport from the endoplasmic reticulum to the Golgi) and then exposed to either heat shock or control conditions as described above (BFA remained in the cell culture medium for the duration of the experiment). Treatment with BFA had no effect on the intracellular HSP70 concentration (Fig. 2A). Furthermore, HSP70 release was unaffected by BFA treatment following control (37 °C) or heat shock (40 or 43 °C) conditions (Fig. 2B). BFA treatment had no effect on LDH release (data not shown). These data support previous findings (2, 3) and demonstrate that in PBMCs the common secretory pathway does not play a role in either basal or heat shock-induced HSP70 release.

It has recently been shown that lipid rafts play a role in heat shock-induced HSP70 release from epithelial cells (3). Cholesterol is the major lipid component of lipid rafts and is required for maintaining raft stability. MBC is a pharmacological agent that specifically depletes cellular cholesterol and consequently causes a disruption to raft functioning (22). Therefore, to examine whether lipid rafts were involved in HSP70 release from PBMCs, PBMCs were treated with MBC (5 or 10 mM for 1 h) and then exposed to either heat shock or control conditions as described above (following 1 h of MBC treatment, PBMCs were resuspended in MBC-free cell culture medium for the duration of the experiment). MBC treatment had little affect on intracellular HSP70 at 37 or 40 °C; however, PBMCs exposed to 43 °C for 1 h in the presence of either 5 or 10 mM MBC had a significantly attenuated intracellular HSP70 response (Fig. 2C). Interestingly, we observed no affect of MBC treatment on the release of HSP70 following either control (37 °C) or heat (40 °C) shock conditions (Fig. 2D). Furthermore, despite the marked attenuation of intracellular HSP70 accumulation at 43 °C by MBC treatment, no affect of MBC was observed on HSP70 release following heat shock at 43 °C (Fig. 2D). To confirm the efficacy of our MBC treatment regimen, we determined the cellular cholesterol concentration in PBMCs treated with either 5 or 10 mM MBC for 1 h or that were untreated (control). MBC treatment was highly effective in removing cholesterol with 5 and 10 mM MBC causing a reduction in cellular cholesterol content by 35 and 65% respectively, compared with untreated control cells (Fig. 2E). These data do not support a role for lipid rafts in either basal or heat shock-induced HSP70 release from PBMCs.

Interestingly, treatment of PBMCs with 10 mM MBC for 1 h resulted in an ~2-fold increase in LDH release compared with untreated cells (Fig. 2F). In the study by Broquet et al. (3), Caco-2 epithelial cells were treated with 10 mM MBC for 6 h. To examine the possibility that the inhibitory effects of MBC treatment on HSP70 release observed by Broquet...
et al. (3) may be the result of nonspecific, cytotoxic effects, PBMCs were pre-incubated with 10 mM MBC for 1 h and then exposed to heat shock (40 or 43 °C) or control conditions (37 °C) for 1 h followed by 4 h of recovery at 37 °C, after which samples were prepared for determination of HSP70 and LDH as described under “Experimental Procedures.” As in the study by Broquet et al. (3), cells were incubated with MBC for the duration of the experiment (6 h). As expected, exposure of PBMCs to 10 mM MBC for 6 h resulted in a marked increase in the release of LDH (6-fold compared with untreated cells), suggesting that this treatment was highly cytotoxic (Fig. 3C). Consistent with our previous data, heat shock (43 °C) resulted in an increase in both the intracellular and extracellular HSP70 concentrations compared with 37 °C (Fig. 3C, A and B, Control). Interestingly, although treatment of PBMCs with 10 mM MBC for 6 h resulted in a total ablation of intracellular HSP70, the extracellular HSP70 concentration was increased, relative to control PBMCs, in both 37 and 43 °C conditions (Fig. 3B). These data demonstrate that exposure of PBMCs to cytotoxic levels of MBC results in an increase in the extracellular HSP70 concentration, most likely because of the MBC-induced degradation in plasma membrane integrity and subsequent “leaking” of HSP70 into the extracellular environment.

An Exosome-dependent Pathway Contributes to the Release of HSP70 from PBMCs in Both Basal and Stress-induced Conditions—It has been hypothesized previously that exosomes may provide a vehicle by which cells can actively release HSP70 (17). Therefore, to examine whether exosomes play a role in HSP70 secretion in both the basal and stress-induced state, we exposed PBMCs to heat shock (1 h at 43 °C followed by 4 h at 37 °C) or control (5 h at 37 °C) conditions. Exosomes were purified according to the method of Savina et al. (20), and exosomal HSP70 content was determined by enzyme-linked immunosorbent assay. To confirm the validity of the exosome isolation procedure, a series of exosome standards were created. FBS contains endogenous exosomes; therefore, FBS was diluted in RPMI 1640 cell culture medium to obtain final FBS concentrations of 20, 10, 5, 2.5, and 0%. Exosomes were isolated as described under “Experimental Procedures.” Exosome-dependent Trafficking of HSP70

**Fig. 2.** The effect of BFA and MBC on HSP70 release. PBMCs were incubated with either BFA (A and B, n = 5) or MBC (C, D, and F, n = 7) for 1 h and then exposed to heat shock (40 or 43 °C) or control conditions (37 °C) for 1 h followed by 4 h of recovery at 37 °C, after which samples were prepared for determination of HSP70 and LDH as described under “Experimental Procedures.” (E, n = 6) PBMCs were incubated with MBC for 1 h, after which samples were prepared for determination of intracellular cholesterol as described under “Experimental Procedures.” Results are expressed as the mean ± S.E. of n independent experiments performed in duplicate. *, p < 0.05 versus 37 (RM ANOVA, main effect); †, p < 0.05 versus Control (Student’s t test); ¶, p < 0.05 versus 5 mM MBC (Student’s t test); ‡, p < 0.05 versus Control (RM ANOVA, main effect).
enzyme specific to exosomes (Fig. 4A). Exosomes are released in the basal state; therefore, to further validate our exosome isolation procedure we purified exosomes from PBMCs cultured for 1, 3, or 5 h. As expected, the greater the duration of the PBMC culture period, the greater the acetylcholinesterase activity we observed, i.e. the greater the number of exosomes secreted (Fig. 4B). A number of proteins are found in exosomes (23) including several HSPs, i.e. HSP90, HSP90α, and HSC70.

To examine whether HSP70 is also expressed in exosomes, purified exosomes from PBMCs cultured for 1, 3, or 5 h were probed with an anti-HSP70 antibody. HSP70 was robustly detectable in exosomes isolated at each time point (Fig. 4C).

To examine whether exosomes play a role in the release of HSP70 from stressed cells, PBMCs were exposed to heat shock (40 or 43 °C for 1 h followed by a 4-h recovery at 37 °C) or control (37 °C for 5 h) conditions and exosomes were purified as described previously. We hypothesized that heat shock may influence HSP70 release via exosomes in three ways, 1) heat shock may augment the exosome secretory rate, 2) heat shock may augment HSP70 content per exosome, and 3) heat shock may augment HSP70 release via a combination of these processes. Although we observed no significant differences in the number of exosomes secreted in response to heat shock (Fig. 4D), the amount of HSP70 within isolated exosomes was significantly greater in heat-shocked PBMCs compared with control (Fig. 4E).

**DISCUSSION**

In the current series of experiments we have demonstrated a novel secretory pathway for HSP70 cellular export. Our data show that HSP70 is expressed within exosomes and that exosomes contribute to the release of HSP70 from PBMCs in both the basal and stress-induced state. Although heat shock had no effect on the exosome secretory rate, exosomal HSP70 content was increased in heat-shocked cells. In addition, our data do not support a role for either the common cellular secretory pathway or lipid rafts in the etiology of stress protein export from PBMCs into the extracellular environment.

Consistent with previous data (2, 3, 5), we found that the release of HSP70 from PBMCs occurs by a mechanism other than the common secretory pathway, because BFA, an inhibitor of protein transport via the endoplasmic reticulum-Golgi, did not influence HSP70 release. However, and in contrast to previous work (3), our results do not support a role for lipid rafts in HSP70 release from PBMCs. In previous studies (3, 5), the involvement of lipid rafts in HSP70 secretion has been examined by treating cells with the lipid raft-disrupting drug methyl-β-cyclodextrin. The efficacy of MBC in disrupting lipid raft integrity is dependent on its ability to remove cellular cholesterol, an integral component of lipid rafts essential to the maintenance of raft stability. In our experiments, treatment of PBMCs with MBC, at concentrations that potently remove cellular cholesterol, had no effect on HSP70 release in either the basal or stress-induced state.

Treatment of PBMCs with MBC at 10 mM for 6 h (an identical concentration and exposure period to that used in the study by Broquet et al. (3)) was cytotoxic. Therefore, we hypothesized that the previously observed inhibitory effect of MBC on HSP70 release (3) may be a consequence of MBC cytotoxicity. However, our results clearly demonstrate that HSP70 release from PBMCs treated with 10 mM MBC for 6 h was greater than in untreated control cells. It is likely that this increase in HSP70 release is as a consequence of the MBC-induced degradation in plasma membrane integrity, resulting in the subsequent leaking of HSP70 from the cytosol into the extracellular environment.

Interestingly, it was recently demonstrated that MBC inhibited the secretion of HSP70 from PBMCs, and, therefore, it was suggested that lipid rafts are involved in this process (5). The concentrations of MBC typically used to disrupt lipid raft function, i.e. remove cellular cholesterol, are 3–10 mM. Indeed, in preliminary experiments, we have demonstrated that 1 mM MBC treatment results in a very small (~5%) decrease in PBMC cholesterol levels. Given that the lipid raft-disrupting effects of MBC are as a consequence of the depletion of cellular cholesterol and that the concentration of MBC used in the study by Hunter-Lavin et al. (5) was ~0.02 mM (cellular cholesterol levels were not determined in this study), the conclusion that lipid rafts play a role in HSP70 release from PBMCs is, based on these data, unsubstantiated.

Based on our findings, and those of Broquet et al. (3), it would therefore appear that distinct exocytotic processes operate in

**Fig. 3.** The effect of prolonged MBC treatment on HSP70 release. PBMCs (n = 5) were incubated with 10 mM MBC or vehicle for 1 h and then exposed to heat shock (43 °C) or control conditions (37 °C) for 1 h followed by 4 h of recovery at 37 °C, after which samples were prepared for determination of HSP70 and LDH as described under "Experimental Procedures." Results are expressed as the mean ± S.E. of n independent experiments performed in duplicate. †, p < 0.05 versus 37 (RM ANOVA, interaction effect); *, p < 0.05 versus Control (RM ANOVA, main effect).

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Based on our findings, and those of Broquet et al. (3), it would therefore appear that distinct exocytotic processes operate in
different cell types to facilitate the release of HSP70. Indeed, it is known that exosomes are secreted from several hematopoietic cells, e.g. T- and B-lymphocytes, dendritic cells, macrophages, and platelets (24). Given the cellular ubiquity of HSP70 release, PBMCs, epithelial cells (3), myotubes, adipocytes, and glia (4) have all been shown to release HSP70, future studies will be required to confirm the involvement of specific exocytotic pathways in HSP70 release from different cell types.

Although heat shock at 40 °C for 1 h resulted in a moderate increase (~75%) in HSP70 release, heat shock at 43 °C for 1 h resulted in a marked increase (~400%) in HSP70 release, compared with control (37 °C) (Fig. 1F). Interestingly, although we observed a large increase in exosomal HSP70 content at 40 °C, compared with 37 °C, heat shock at 43 °C resulted in a relatively small further increase in exosomal HSP70 content compared with heat shock at 40 °C (Fig. 4E). Collectively, these data suggest that although exosomes contribute to the release of HSP70 in the basal (37 °C) and stress-induced (40 and 43 °C) state, an additional mechanism is likely to play a role in HSP70 release at 43 °C. In the present series of studies we have assessed LDH release to quantify the rate of cell death. Our data clearly show that heat shock per se does not influence LDH release. However, it is important to note that cell death does occur in a low percentage of cells over the duration of the culture period. We hypothesize that the large increase in HSP70 release from PBMCs exposed to 43 °C for 1 h may be related, in part, to the marked increase in the intracellular HSP70 level and the subsequent leaking of HSP70 into the extracellular milieu via a simple mass-action effect. Future studies will be required to examine this hypothesis.

Our studies do not identify the functional consequence of HSP70 release via exosomes. However, recent studies suggest an important role for exosomes in the modulation of immune responses. Specifically, exosomes purified from B-lymphocytes (25) and dendritic cells (19) are enriched in MHCII and T-cell co-stimulatory molecules and stimulate T-lymphocytes. These data have led to the suggestion that secreted exosomes expressing relevant peptide-MHC complexes may play a role in the generation of immune responses in vivo (26). Interestingly, the treatment of dendritic cells with HSP70 up-regulates the expression of MHCII and T-lymphocyte co-stimulatory

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**Fig. 4. Role of exosomes in basal and stress-induced HSP70 release.** A (n = 3), exosomes were purified from 20, 10, 5, 2.5, and 0% FBS in RPMI 1640 cell culture medium, after which acetylcholinesterase activity was determined as described under “Experimental Procedures.” B and C (n = 3), PBMCs were incubated for 1, 3, or 5 h at 37 °C, after which exosomes were purified and prepared for determination of acetylcholinesterase activity and HSP70 as described under “Experimental Procedures.” D and E (n = 6), PBMCs were exposed to heat shock (43 °C) or control conditions (37 °C) for 1 h followed by 4 h of recovery at 37 °C, after which exosomes were purified and prepared for determination of acetylcholinesterase activity and HSP70 as described under “Experimental Procedures.” Results are expressed as the mean ± S.E. of n independent experiments performed in duplicate. *, p < 0.05 versus 37 (Student’s t test); †, p < 0.05 versus 40 (Student’s t test).
molecules (11) and induces potent, T-lymphocyte-mediated, antitumor immunity (27). Given that 1) HSP70 and purified exosomes have a considerable degree of functional homology, and 2) HSP70 is abundantly expressed within secreted exosomes, we suggest that the immunogenicity of secreted exosomes may, in part, be because of the presence of HSP70.

An intriguing aspect of the present work is how is HSP70 targeted to exosomes? MVs, the organelles from which exosomes are derived, are generated from the fusion of early endosomes and have a well established role in the degradation of proteins internalized from the cell surface via fusion with lysosomes (18). However, in addition to fusion with lysosomes, MVs are able to undergo exocytotic fusion with the plasma membrane and release their “intraluminal vesicles” (the term for exosomes while they are contained within the MVb) (24). Interestingly, HSP70 is highly expressed on the cell surface (14, 17), raising the possibility that cell surface-bound HSP70 is initially internalized via early endosomes and subsequently incorporated into MVb before being secreted via the fusion of MVb with the plasma membrane.

On the basis of early findings demonstrating that exosomes mediate tumor rejection (19), exosomes isolated from monocyte-derived dendritic cells have been tested for the immunotherapy of cancer patients (26). Exosomes contain many proteins essential to the generation of immune responses, e.g., MHCI and MHCII, CD81, CD86, and CD11b, and, as discussed above, the presence of HSP70 within exosomes may also contribute to exosome immunogenicity. Given the known immunostimulatory properties of HSP70, and our data demonstrating that the HSP70 content of exosomes is significantly increased following a transient heat shock, it is an intriguing possibility that the immunogenicity of clinical grade exosomes for use in cancer vaccines may be enhanced by exposing cells to transient, sublethal heat shock.

In conclusion, we show for the first time that exosomes contribute to HSP70 release from PBMCs in both the basal (37 °C) and stress-induced (40 or 43 °C for 1 h) state. Furthermore, although lipid rafts contribute to the release of HSP70 from heat-shocked epithelial cells, our results suggest that lipid rafts do not contribute to HSP70 release from PBMCs. Given the known immunomodulatory functions of both recombinant HSP70 and purified exosomes and that HSP70 is highly expressed within exosomes, we suggest that exosomal HSP70 may account for some of the immunostimulatory properties of exosomes.

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