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Dual function of membrane-bound heat shock protein 70 (Hsp70), Bag-4, and Hsp40: protection against radiation-induced effects and target structure for natural killer cells

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Abstract

CX+/CX− and Colo+/Colo− tumor sublines with stable heat shock protein 70 (Hsp70) high and low membrane expression were generated by fluorescence activated cell sorting of the parental human colon (CX2) and pancreas (Colo357) carcinoma cell lines, using an Hsp70-specific antibody. Two-parameter flow cytometry revealed that Hsp70 colocalizes with Bag-4, also termed silencer of death domain, not only in the cytosol but also on the plasma membrane. After nonlethal γ-irradiation, the percentage of membrane-positive cells and the protein density of Hsp70 and Bag-4 were found to be strongly upregulated in carcinoma sublines with initially low expression levels (CX−, Colo−). Membrane expression of Hsp70 was also elevated in Bag-4 over-expressing HeLa cervix carcinoma cells when compared to neo-transfected cells. In response to γ-irradiation, neo-transfected HeLa cells behaved like Hsp70/Bag-4 low-expressing CX− and Colo−, and Bag-4-transfected HeLa cells like Hsp70/Bag-4 high-expressing carcinoma sublines CX+ and Colo+. Immunoprecipitation studies further confirmed colocalization of Hsp70 and Bag-4 but also point to an association of Hsp70 and Hsp40 on the plasma membrane of CX+ and Colo+ cells; on CX− and Colo− tumor sublines, Hsp40 was detectable in the absence of Hsp70 and Bag-4. Other co-chaperones including Hsp60 and Hsp90 were neither found on the cell surface of CX+/CX−, Colo+/Colo− nor on HeLa neo-/HeLa Bag-4-transfected tumor cells. Functionally, Hsp70/Bag-4 and Hsp70/Hsp40 membrane-positive tumor cells appeared to be better protected against radiation-induced effects, including G2/M arrest and growth inhibition, on the one hand. On the other hand, membrane-bound Hsp70, but neither Bag-4 nor Hsp40, served as a recognition site for the cytolytic attack mediated by natural killer cells.
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Keywords: G2/M arrest; γ-irradiation; Hsp70/Bag-4/Hsp40 membrane expression; NK cells

Abbreviations: ATP, adenosine 5′-triphosphate; Bag, Bcl-2-associated athanogene; DR3, death receptor 3; FITC, fluorescein isothiocyanate; HGF, hepatocyte growth factor; Hip, heat shock protein interacting protein; HSP, heat shock protein family; Hsp70, heat shock protein 70 family member; NK cell, natural killer cell; PDGF, platelet-derived growth factor; Raf, serine/threonine protein kinase; SODD, silencer of death domain; TNF, tumor necrosis factor; TNFR1, TNF receptor 1

Introduction

Heat shock proteins (HSPs) are highly conserved molecules known to mediate protection against lethal damage after a variety of different stress stimuli in procaryotic and eucaryotic cells. Also under physiological conditions, they support folding of non-native and misfolded proteins, and prevent aggregation during proliferation and cellular differentiation.[1] One of the best-characterized chaperones belong to the HSP70 family. Similar to other stress proteins, HSP70s are most efficient when they operate in concert with co-chaperones including HSP40s, HSP60s, and HSP90s. Together with J-domain chaperones, they support protein folding and assist translocation processes across membranes.[2] Adenosine 5′-triphosphate (ATP) hydrolysis, stabilizing HSP70 substrate complexes, is much faster in collaboration with the HSP-interacting protein Hip.[3] Apoptosis initiated either by exogenous factors (i.e. tumor necrosis factor α, TNFα) or spontaneous crosslinking of the death domain receptors TNF receptor 1 (TNFR1), death receptor 3 (DR3) is inhibited in the presence of high amounts of Hsc70 and Hsp70,[4] in combination with members of the antiapoptotic Bcl-2-associated athanogene (BAG) family. They share a highly conserved 45 aa BAG domain consisting of three-helix bundles of variable length and a diverse N-terminal sequence.[5] Four BAG proteins (Bag-1, -3, -4, and -6) have been reported to compete with Hip for binding to the ATPase domain of HSP70s, and thus promote chaperone activity.[6,7] It

was also assumed that BAG proteins operate as cellular adaptors targeting HSP70/BAG complexes to the cytosolic domain of the 55 kDa TNFR1, and thereby inhibit receptor aggregation and activation of the death domains via TRADD, FADD, TRAF and RIP.[8] On the one hand, overexpression of Bag-4, also termed as the silencer of death domain (SODD), has been found to suppress TNF-induced apoptosis[9] and, on the other hand, in mice, increased HSP70 levels are known to confer protection against TNF-mediated lethal shock.[10] BAG proteins were also discussed as important cofactors, affecting the ATPase cycle of HSP70s.[9] The availability of hydrolyzable ATP regulates BAG binding. Apart from TNFR1, BAG proteins interact with Bcl-2,[11] serine/threonine protein kinase (Raf kinase),[12] androgen-,[13] hepatocyte growth factor (HGF)-, and platelet-derived growth factor (PDGF) receptors.[14] Following stress, when cytosolic HSP70 levels are upregulated, the BAG/receptor complexes might be replaced by BAG/HSP70 complexes.

In the present study, we compared the interaction of the stress-inducible Hsp70 and Bag-4 under physiological conditions and after γ-irradiation-induced stress in carcinoma cells with initially different Hsp70 membrane expression pattern. Although the molecular basis for the interaction between the ATPase domain of Hsp70 and the short BAG domain of Bag-4 has been studied extensively,[13,15] knowledge on stress-induced modulations in the plasma membrane is limited. Previously, we and others demonstrated a tumor-selective Hsp70 plasma membrane localization by cell surface iodination followed by SDS–PAGE and by flow cytometry of viable tumor cells, using an Hsp70-specific monoclonal antibody.[16,17] These findings are in line with recently published data,[18] showing an abundance of molecular chaperones, including Hsp70, in the plasma membrane of tumor cells by global proteome analysis of surface-bound proteins. In this context, we were also interested to identify other co-chaperones including Hsp40, Hsp60, and Hsp90 that might be associated with Hsp70/Bag-4 on the cell membrane.

We have also shown that the amount of membrane-bound Hsp70 on tumor cells could be modulated by membrane-interactive reagents[19] and cytostatic drugs.[20] Also, radiation has been found to affect cytosolic Hsp70 levels in tumor cells.[21–23] Since γ-irradiation is frequently used in cancer therapy,[24,25] it is important to understand the molecular nature of radiation-induced effects on resistance of tumor cells to apoptosis.[26,27] Therefore, we investigated radiation-induced effects in three independent carcinoma cell systems that differ with respect to their capacity to express Hsp70 on their cell surface. Concomitantly, the effects of Bag-4 as an Hsp70-interacting, antiapoptotic molecule were investigated. We found that Hsp70 was associated with Bag-4 and with Hsp40 not only in the cytosol but also on the plasma membrane. Following nonlethal γ-irradiation, the chaperone complex was found to be upregulated in carcinoma cells with initially low Hsp70 expression levels. On the one hand, functionally, an Hsp70/Bag-4/Hsp40-positive phenotype conferred protection against irradiation-induced effects including G2/M arrest and growth inhibition and, on the other hand, Hsp70, but not associated proteins, served as a recognition structure for the cytolytic attack mediated by natural killer (NK) cells.

Results

γ-Irradiation induces an increase in membrane-bound Hsp70 and Bag-4 in initially low-expressing carcinoma sublines

By cell sorting of the parental CX2 colon and Colo357 pancreas carcinoma cell lines, using the Hsp70-specific monoclonal antibody cmHsp70.1 (Table 1), stably Hsp70 high- (CX+: >80%; Colo+: >75%) and low-expressing (CX−: <30%; Colo−: <35%) tumor sublines were generated. To determine the nonlethal irradiation dose, exponentially growing carcinoma sublines with initially different Hsp70 membrane expression pattern were irradiated, either fractionated (2×2 and 5×2 Gy), or with a single dose of 1×10 or 1×20 Gy, respectively. Up to a total irradiation dose of 1×10 Gy, and a recovery period of 24 h at 37°C, cell viability of colon and pancreas carcinoma sublines remained unaffected (Table 2a). γ-Irradiation at 1×20 Gy resulted in a significant loss of cell viability in Hsp70 low-expressing CX− and Colo− tumor cells (Table 2a). Viability of HeLa cervix carcinoma cells transfected with neo vector was only marginally reduced after irradiation of 1×20 Gy (Table 2a). Annexin-V–fluorescein isothiocyanate (FITC) staining confirmed the data obtained by Trypan blue exclusion assays. None of the tumor sublines showed significant increase in Annexin-V–

Table 1 List of antibodies used for fluorescence-activated CS, WB analysis FC, IP, and ABS

Antibody	Specificity	Recognition site	Assay
Poly/monoclonal, isotypic control	Isotype	Not determined	FC
Monoclonal mouse IgG1, cmHsp70.1	Hsp70	C-terminus, $aa_{450–463}$	CS, WB, FC, ABS
Polyclonal rabbit Ig, H-300	Bag-4	C-terminus, $aa_{158–457}$	WB, FC, ABS
Polyclonal rabbit Ig, IMG-152	Bag-4	C-terminus, $aa_{443–457}$	WB, FC, ABS
Polyclonal goat Ig, N-19	Bag-4	N-terminus	WB, FC, ABS
Polyclonal rabbit Ig, FL-274	Bag-1, p50	Full length, $aa_{1–274}$	WB, FC, ABS
Polyclonal goat Ig, C-16	Bag-1, p32, p36, p50	C-terminus	WB, FC, ABS
Monoclonal mouse IgG2a, SPA-450 (2E1)	Hsp40	J domain, $aa_{1–70}$	IP, ABS
Polyclonal rabbit Ig, SPA-400	Hsp40	Full length, $aa_{1–340}$	FC, IP, ABS
Monoclonal mouse IgG1, SPA-806	Hsp60	C-terminus, $aa_{383–447}$	FC, IP, ABS
Monoclonal mouse IgG1, SPA-830	Hsp90	C-terminus, $aa_{604–697}$	FC, IP, ABS
Monoclonal mouseIgG1, Ab-1	Tubulin, p60	Not determined	WB

CS: cell sorting; WB: Western blot; FC: flow cytometry; IP: immunoprecipitation; ABS: antibody blocking studies

Table 2a Viability (%) of tumor cells following single or fractionated γ-irradiation as determined by Trypan blue exclusion

Tumor	Control	2 × 2 Gy	5 × 2 Gy	1 × 10 Gy	1 × 20 Gy
CX+	100±0.0[a]	100±0.0	97.8±2.1	96.6±0.0	95.0±2.8
CX−	100±0.0	96.6±0.9	96.0±0.1	98.6±0.0	87.5*±0.02 ($P<0.02$)
Colo+	100±0.1	NT	NT	97.0±0.7	88.9±0.6
Colo−	100±0.0	NT	NT	90.8±1.2	80.5*±5.3 ($P<0.02$)
HeLa Bag-4	100±0.1	NT	NT	97.7±1.3	96.7±1.9
HeLa neo	100±0.0	NT	NT	98.0±0.1	91.4±0.6

On day 2 after cell passage, exponentially growing, adherent carcinoma cells were irradiated with different doses as indicated. Following a 24 h recovery period at 37°C, cell viability was determined by Trypan blue exclusion assays. Data represent mean values of viable cell counts derived from three independent experiments; NT, not tested; *Values significantly different from control ($P<0.02$) [a]% viable cells

Table 2b Percentage of Annexin-V–FITC positively stained, apoptotic tumor cells following single γ-irradiation

Tumor	Control	1 × 10 Gy	*n*
CX+	11.8±2.3	11.6±1.3	5
CX−	17.4±9.2	19.2±7.0	5
Colo+	18.5±4.4	24.8±7.8	6
Colo−	17.2±3.7	25.0±2.5	6
HeLa Bag-4	17.0±5.6	10.0±5.6	4
HeLa neo	10.0±5.1	12.9±8.7	4

On day 2 after cell passage, exponentially growing, adherent carcinoma cells were irradiated with a single dose of 1 × 10 Gy. Following a 24 h recovery period at 37°C, apoptotic cell death was determined by Annexin-V–FITC staining. The data represent mean values of indicated numbers of experiments±S.E.: none of the data is significantly different ($P<0.05$) as compared to control values

FITC-positive apoptotic cells after treatment with 1 × 10 Gy (Table 2b), whereas irradiation at 1 × 20 Gy resulted in significant apoptosis (Nylandsted J, Gyrd-Hansen M, Danielewicz A, Fehrenbacher N, Lademann U, Hoyer-Hansen M, Weber E, Multhoff G, Rohde M and Jaattela M (2004) Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. J. Exp. Med. 200: 425–435). With respect to these findings, all further experiments were performed with the nonlethal γ-irradiation dose of 1 × 10 Gy, followed by a recovery period of 24 h at 37°C.

Although the carcinoma sublines CX+/CX− and Colo+/Colo− differed significantly in their capacity to express Hsp70 on the cell surface, the cytosolic Hsp70 content was comparable in both tumor subline systems. Even after nonlethal γ-irradiation, the cytosolic Hsp70 content remained unaltered, as determined by quantitative Western blot analysis related to tubulin (Figure 1a and b, upper part).

It is well known that Bag-4 interacts with the ATPase domain of Hsp70 in the cytosol.[13,15] Therefore, in addition to Hsp70, the cytosolic amount of Bag-4 was measured in untreated and γ-irradiated colon (CX+/CX−) and pancreas (Colo+/Colo−) carcinoma sublines, using the Bag-4-specific antibodies H-300 and IMG-152 (Table 1) and related to tubulin. Epithelial carcinoma were described to have elevated Bag-4 expression levels,[28] conferring resistance to TNF-induced cell death. Our data are in line with these findings; the relative protein amount of Bag-4 (Figure 1, lower part) was found to be elevated when compared to that of Hsp70 (Figure 1, upper part) in colon and pancreas carcinoma sublines.

Identical to Hsp70, following nonlethal γ-irradiation at 1 × 10 Gy, the cytosolic Bag-4 content remained unaltered in all tumor sublines (Figure 1a and b, lower part). Comparable results were observed with respect to Bag-1, using antibodies FL-274 and C-16 (Table 1) directed against full-length, p50, p36, and p32, respectively, in untreated and irradiated (1 × 10 Gy) CX+ and CX− tumor sublines (data not shown).

Although, the cytosolic amount of Hsp70 and Bag-4 remained unaltered after nonlethal γ-irradiation, the cell surface expression pattern of Hsp70 was analyzed. As indicated in Figure 2a (upper graph), the percentage of Hsp70 membrane-positive cells remained unaltered and high in CX+ carcinoma cells after γ-irradiation (82 *versus* 81%). However, a significant increase in the percentage of Hsp70 membrane-positive cells was determined in CX− carcinoma cells ($P<0.04$); the percentage of Hsp70-positive cells rose from 34 to 59%, indicating a translocation of cytosolic Hsp70 to the plasma membrane. Interestingly, Hsp70 membrane-positive CX+ cells were also found to be strongly positive for Bag-4 (74%, IMG-152; 78%, H-300), as measured by two independent Bag-4 antibodies (Table 3). Both antibodies recognize a sequence in the C-terminal domain of Bag-4 (Table 1). In contrast, a Bag-4, antibody whose epitope is localized in the N-terminus (N-19), and antibodies directed against Bag-1 (FL-274, C-16) were unable to detect plasma membrane-bound BAG molecules on viable tumor cells (Table 3).

Only 42% of the Hsp70 low-expressing CX− cells exhibited a Bag-4-positive phenotype (Figure 2a, lower graph). Similarly, a significant elevation in Bag-4 expressing cells (42 to 73%) was predominantly found in CX− cells but not in CX+ cells (78 to 79%). The affinity of the Hsp70- and Bag-4-specific antibodies was identical, as indicated by comparable

mean fluorescence intensity (mfi) values in untreated and treated tumor sublines (Figure 2, right part). After irradiation, a significant increase in the protein density of Hsp70 ($P<0.03$) and Bag-4 ($P<0.02$) proteins was detected in CX− cells

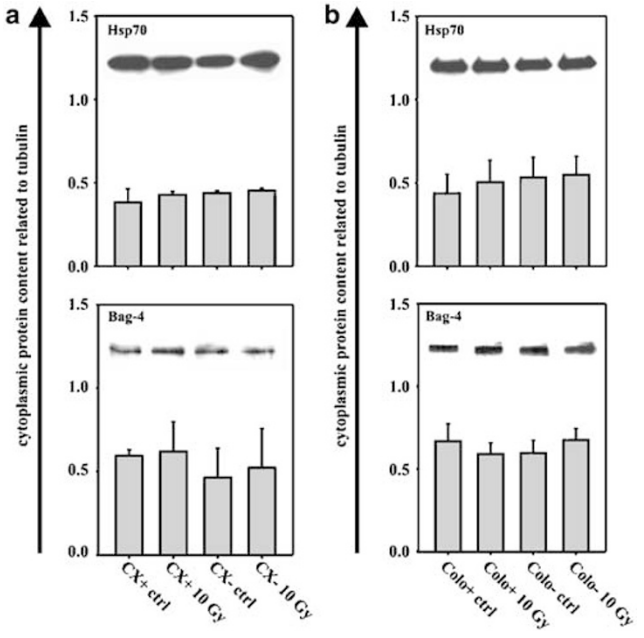


Figure 1 Cytosolic Hsp70 and Bag-4 levels remained unaltered in colon (CX + /CX−) and pancreas (Colo + /Colo−) carcinoma sublines after nonlethal γ-irradiation. Adherent growing CX + /CX− (**a**) and Colo + /Colo−(**b**) carcinoma cells were kept either untreated (ctrl) or were irradiated (1 × 10 Gy). After a recovery period of 24 h, cell lysates were prepared, and equal protein amounts (10 μg) were run on a 10% SDS–PAGE and transferred to nitrocellulose membranes. Western blots were stained with Hsp70 (cmHsp70.1)- and Bag-4 (H-300)-specific antibodies. Characteristics of the different antibodies are summarized in Table 1. Data show one representative Western blot analysis and mean values as histograms of four independent experiments ± S.E.; none of the values was statistically different from control, as determined by the Student's *t*-test

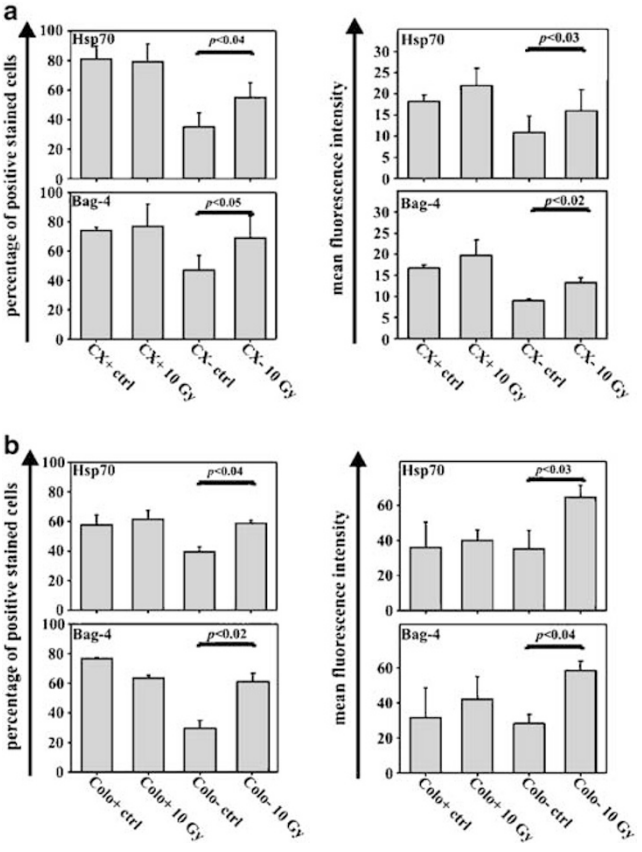


Figure 2 Cell surface expression of Hsp70 and Bag-4 was upregulated on Hsp70 low-expressing colon (CX−) and pancreas (Colo−) carcinoma sublines after nonlethal γ-irradiation. The percentage of positively stained cells (left part) and the mean fluorescence intensity (right part) of Hsp70 and Bag-4 were determined by flow cytometry on untreated (ctrl) and γ-irradiated (1 × 10 Gy) colon (**a**: CX + /CX−) and pancreas (**b**: Colo + /Colo−) carcinoma cells, using Bag-4-specific antibodies recognizing the C-terminal localized BAG domain (Table 1). Histograms represent mean values of eight (Hsp70) and seven (Bag-4) independent experiments ± S.E.; significant differences are indicated as *P*-values in each histogram

Table 3 Percentage of positively stained tumor cells using different antibodies directed against Bag-1, Bag-4, Hsp70, Hsp40, Hsp60, and Hsp90

Tumor Antibody	CX+	CX−	Colo+	Colo−	HeLa Bag-4	HeLa neo
Bag-1 C-16	0.5 ± 0 ($n=3$)	2.3 ± 1.3 ($n=3$)	1.0 ± 1.0 ($n=3$)	1.5 ± 0.5 ($n=3$)	NT	NT
Bag-1 FL-274	2.9 ± 0.2 ($n=3$)	2.2 ± 0.2 ($n=3$)	2.0 ± 2.3 ($n=3$)	1.8 ± 0.5 ($n=3$)	NT	NT
Bag-4 N-19	2.4 ± 1.6 ($n=3$)	0 ± 0 ($n=3$)	0 ± 0 ($n=3$)	1.0 ± 0.5 ($n=3$)	NT	NT
Bag-4 IMG-152	74 ± 2.5 ($n=3$)	47 ± 10 ($n=3$)	77 ± 2.9 ($n=3$)	26 ± 5.2 ($n=3$)	75 ± 4.7 ($n=3$)	49 ± 2.5 ($n=3$)
Bag-4 H-300	78 ± 2.2 ($n=6$)	42 ± 10 ($n=6$)	78 ± 0.7 ($n=6$)	29 ± 11.3 ($n=6$)	78 ± 4.7 ($n=6$)	49 ± 5.5 ($n=6$)
Hsp70 cmHsp70.1	82 ± 8.8 ($n=6$)	34 ± 9.5 ($n=6$)	60 ± 9.7 ($n=6$)	37 ± 3.1 ($n=6$)	68 ± 11.4 ($n=6$)	38 ± 5.5 ($n=6$)
Hsp40 SPA-400	17 ± 5.5 ($n=6$)	23 ± 3.8 ($n=7$)	15 ± 6.5 ($n=4$)	13 ± 5.1 ($n=6$)	14 ± 0.8 ($n=4$)	19 ± 3.4 ($n=4$)
Hsp60 SPA-806	1.7 ± 0.0 ($n=3$)	1.1 ± 0.15 ($n=3$)	1.4 ± 0.15 ($n=3$)	1.4 ± 0.6 ($n=3$)	0.7 ± 0.3 ($n=3$)	2.2 ± 0.4 ($n=3$)
Hsp90 SPA-830	2.6 ± 0.45 ($n=3$)	2.1 ± 0.7 ($n=3$)	1.7 ± 0.3 ($n=3$)	1.7 ± 0.6 ($n=3$)	1.8 ± 0.5 ($n=3$)	3.1 ± 1.3 ($n=3$)

Data represent mean values of at least three independent experiments ± S.E.: <5% was evaluated as negative: NT, not tested

(Figure 2a, left part). Regarding these results, we concluded that nonlethal γ-irradiation results in an upregulated Hsp70 and Bag-4 membrane expression in carcinoma sublines with initially low expression levels.

These data could be confirmed in pancreas carcinoma sublines Colo+/Colo−, which were generated using the same protocol as described for CX+/CX− sublines.[29] After cell sorting, about 60% of the Colo+ cells exhibited an Hsp70 membrane-positive phenotype, but only 37% of the Colo− cells were found to be Hsp70 membrane-positive (Table 3). With respect to Bag-4, 77% of the Colo+ cells and 26% of the Colo− cells were membrane-positive, as determined with the antibody IMG-152 (Table 3). This phenotype remained stable in the tumor sublines for at least 30 cell passages. As already shown for CX+/CX−, and in contrast to the differences detected in the cell surface expression pattern, the intracellular Hsp70/Bag-4 content was identical in Colo+/Colo− cells (Figure 1b). After nonlethal γ-irradiation, the percentage of Hsp70 (37–58%, $P<0.04$) and Bag-4 (26–60%, $P<0.02$) increased selectively in Colo− but not in Colo+ carcinoma cells (Figure 2b, left graph). Similar to CX− colon carcinoma cells, also the protein density of Hsp70 ($P<0.03$) and Bag-4 ($P<0.04$) on a single cell level was elevated in Colo− cells (Figure 2b, right graph). No cell surface expression was determined using antibodies directed against the N-terminal region of Bag-4 or against Bag-1 with epitopes residing in the C- or N-terminus (Table 3).

A comparison of the amount of plasma membrane-bound Hsp70 and Bag-4 in untreated and irradiated (1 × 10 Gy) CX+/CX− (Figure 2a) and Colo+/Colo −(Figure 2b) sublines was in line with data from flow cytometry (Figure 2). Again a significant increase in the amount of Hsp70 and Bag-4 was predominantly found in CX− and Colo− tumor sublines after nonlethal irradiation (Figure 3). That of CX+ and Colo+ tumor sublines remained unaltered and high after identical treatment. Regarding these results, derived from two independent carcinoma cell systems, we speculated about a coregulated expression of Hsp70/Bag-4 on the plasma membrane of tumor cells. To further address this question, HeLa cervix carcinoma cells were transfected either with mouse Bag-4, which is highly homologous to human Bag-4, or with neo vector, as a control. The cytosolic amount of Hsp70 and Bag-4 in untreated and irradiated HeLa cells is illustrated in Figure 4. Compared to neo-transfected (Figure 4, lower graph) or untransfected (data not shown) HeLa cells, Bag-4-transfected HeLa cells exhibited a more than six-fold increase in cytosolic Bag-4, if related to tubulin. Nevertheless, this Bag-4 overexpression did not affect cytosolic Hsp70 content (Figure 4, upper graph). Also, after nonlethal γ-irradiation, the cytosolic Hsp70 and Bag-4 levels remained unaltered when compared to nonirradiated cells (Figure 4).

Striking differences were detected with respect to membrane-bound Hsp70 and Bag-4 in the HeLa cell system. As shown in Figure 5 (left part) and Table 3, the number of Hsp70 membrane-positive cells in Bag-4-transfected HeLa cells was 68% and that in neo-transfected cells was 38%. Differences in Bag-4 membrane expression on HeLa cells were as follows: Bag-4-transfected, 78%; neo-transfected, 49%. Following γ-irradiation, a significant increase in the percentage of Hsp70 ($P<0.04$) and Bag-4 ($P<0.001$) positivity was selectively

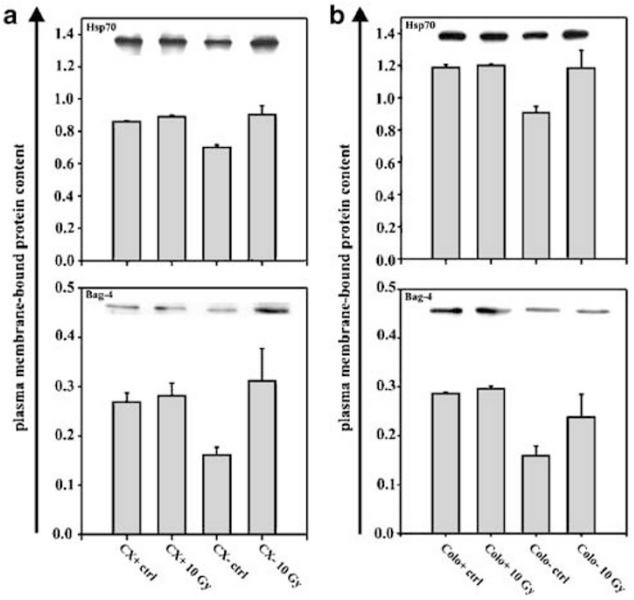


Figure 3 Amount of plasma membrane-bound Hsp70 and Bag-4 increased in CX− and Colo− tumor sublines after nonlethal γ-irradiation. Plasma membranes were purified from untreated (ctrl) and irradiated (10 Gy) CX+/CX−(**a**) and Colo+/Colo− (**b**) tumor sublines (50 × 10^6 cells each). Equal amounts (10 μg) of plasma membrane-bound proteins were subjected to a 10% SDS–PAGE and blotted onto nitrocellulose membranes. One representative Western blot analysis using Hsp70 and Bag-4 antibodies and histograms showing mean values ± S.E. of three independent experiments are illustrated. Increase in the protein amount of Hsp70 and Bag-4 on CX− and Colo− sublines after irradiation was statistically significant ($P<0.05$)

observed in HeLa neo cells (Hsp70: 38–58%; Bag-4: 49–76%). As already shown for CX− and Colo− cells, concomitant with the increase in the percentage of positively stained cells, the protein density per cells of Hsp70 ($P<0.03$) and Bag-4 ($P<0.05$) were also found to be elevated in neo-transfected HeLa cells (Figure 5, right part). Purified plasma membrane preparations of neo-transfected and Bag-4-transfected HeLa cells were in line with these findings (data not shown). Taken together, γ-irradiated HeLa neo cells reacted similar to CX− and Colo− cells with a significant increase in the percentage and protein density of Hsp70/Bag-4 on the cell surface, whereas Bag-4-transfected cells reacted like CX+ and Colo+ cells. It is worth mentioning that untransfected HeLa cells behaved identical to neo-transfected cells (data not shown).

Cell surface expression of Hsp70 and Bag-4 is similarly upregulated in tumor cells with initial low expression levels by γ-irradiation

To test whether upregulation of the Hsp70 and Bag-4 membrane expression might be associated, multiparameter flow cytometry analysis was performed using FITC-conjugated Hsp70 and PE-conjugated Bag-4 antibodies. In Figure 6a–c, horizontal and vertical lines mark the results derived from staining with isotype-matched control antibodies. Under

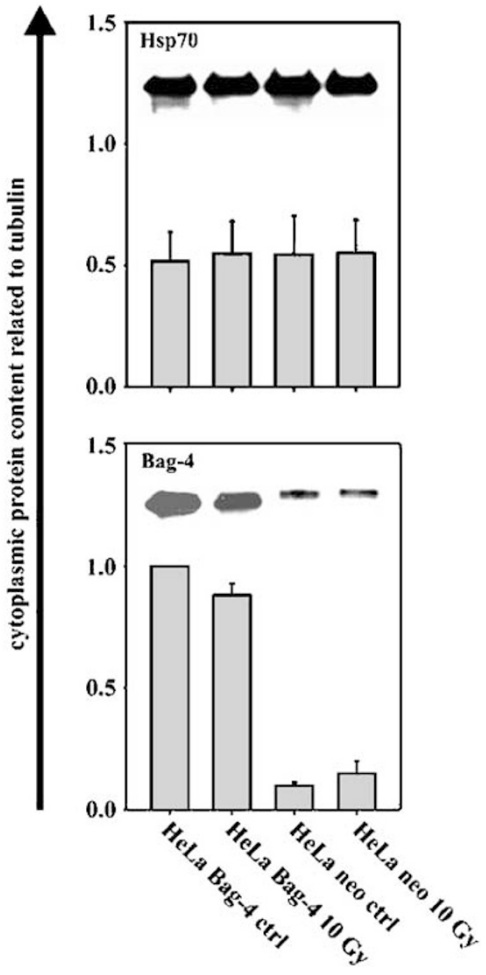


Figure 4 Cytosolic Hsp70 and Bag-4 levels in Bag-4 overexpressing or neo-transfected HeLa cervix carcinoma cells remained unaltered after nonlethal γ-irradiation. HeLa cervix carcinoma cells transfected with mouse Bag-4 revealed a more than six-fold increase in cytosolic Bag-4 levels as compared to neo-transfected or untransfected (data not shown) cells. HeLa Bag-4- and neo-transfected carcinoma cells were maintained either untreated (ctrl) or were irradiated (1 × 10 Gy). After a recovery period of 24 h, cell lysates were prepared and equal protein amounts (10 μg) were run on a 10% SDS–PAGE, and transferred to nitrocellulose membranes. Western blots were stained with Hsp70- and Bag-4-specific antibodies (Table 1). Data show one representative Western blot analysis and histograms of four independent experiments ± S.E., related to tubulin

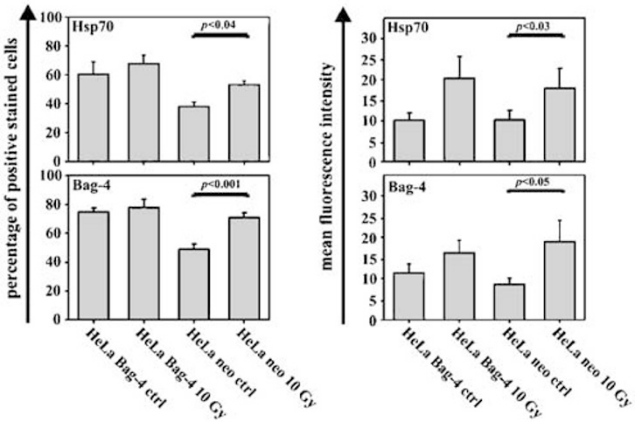


Figure 5 Cell surface expression of Hsp70 and Bag-4 was upregulated on neo-transfected HeLa cells after nonlethal γ-irradiation. The percentage of positive cells (left part) and the mean fluorescence intensity (right part) of Hsp70 and Bag-4 were determined by flow cytometry on untreated (ctrl) and irradiated (1 × 10 Gy) HeLa Bag-4-/HeLa neo-transfected carcinoma cells, using Bag-4-specific antibodies recognizing the C-terminally localized BAG domain (Table 1). Histograms represent mean values of seven independent experiments ± S.E.; significant differences are indicated as *P*-values

physiological conditions (ctrl), an Hsp70/Bag-4 double-positive cell population, in the upper right corner of each graph, was visible only in CX+, Colo+, and HeLa Bag-4-transfected cells (upper part). After nonlethal γ-irradiation (1 × 10 Gy), the percentage of double-positive cells was not elevated significantly in CX+ (Figure 6a: 71–77%), Colo+ (Figure 6b: 65–75%), and HeLa Bag-4-transfected (Figure 6c: 73–74%) carcinoma cells. In contrast, in initially low-expressing CX− (Figure 6a: 35–73%), Colo− (Figure 6b: 34–52%), and HeLa neo-transfected (Figure 6c: 49–73%) cells, the amount of Hsp70/Bag-4 double-positive cells was drastically enhanced after γ-irradiation. This is illustrated in the lower parts of Figure 6a–c (ctrl *versus* 10 Gy) by a shift of the cell population from the lower left corner to the upper right corner. These data confirmed the results obtained by single antibody staining (Figures 2 and 5), and also provide a hint that membrane expression of Hsp70 and Bag-4 might be coregulated in tumor cells with initially low membrane expression levels.

Hsp70 and Bag-4 are colocalized on the cell surface of tumor cells

To further test this hypothesis, immunocytochemistry studies were performed using a two-color overlay staining technique. Figure 7a represents a typical image of a Bag-4 PE single staining in red, and Figure 7b an Hsp70 FITC single staining in green. Orange dots in the overlay of both staining patterns revealed that most Hsp70 and Bag-4 molecules on the plasma membrane are colocalized (Figure 7c). Light microscopic analysis, as indicated in Figure 7d, showed regular cell morphology of the Hsp70/Bag-4 double-positive tumor cells used in these assays. The corresponding isotype-matched negative control staining is shown in Figure 7e–g. A higher magnification of a typical Hsp70/Bag-4 costaining pattern and the corresponding light microscopic view are illustrated in Figure 7i and j.

Hsp70/Bag-4 are associated with Hsp40 on the plasma membrane of CX+ and Colo+ tumor sublines

In the cytosol, Hsp70 frequently operates in concert with several co-chaperones including Hsp40, Hsp60, and Hsp90.[2] To test whether one of these chaperones might be associated with Hsp70/Bag-4 on the cell surface of tumor cells, flow cytometric analyses were performed. As summarized in

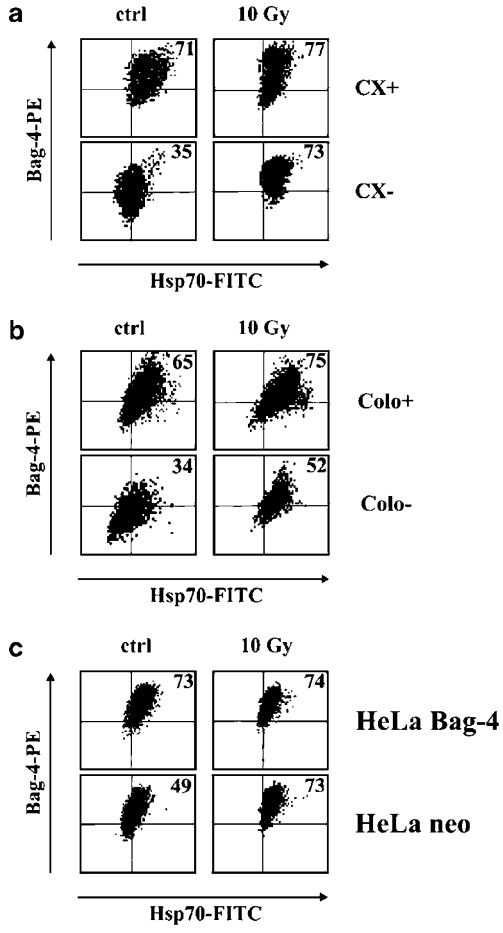


Figure 6 Following nonlethal γ-irradiation, the Hsp70 and Bag-4 double-positive cell population is upregulated in Hsp70/Bag-4 low-expressing carcinoma sublines. Different carcinoma sublines CX+/CX− (**a**), Colo+/Colo− (**b**), and HeLa Bag-4/HeLa neo (**c**), kept either untreated (ctrl) or irradiated (10 Gy), were double-positively stained with Hsp70 FITC and Bag-4 PE and analyzed by multiparameter flow cytometry. Values for isotype-matched control antibodies for each individual cell type, either untreated (ctrl) or after irradiation (10 Gy), are marked as vertical and horizontal lines. The percentages of Hsp70/Bag-4 double-positively stained cells corrected for the nonspecific staining using isotype-matched control antibodies are indicated as numbers in the upper right corner of each graph. Only 7-AAD-negative, viable cells were gated. Dot blots illustrate one representative staining pattern out of three identical experiments

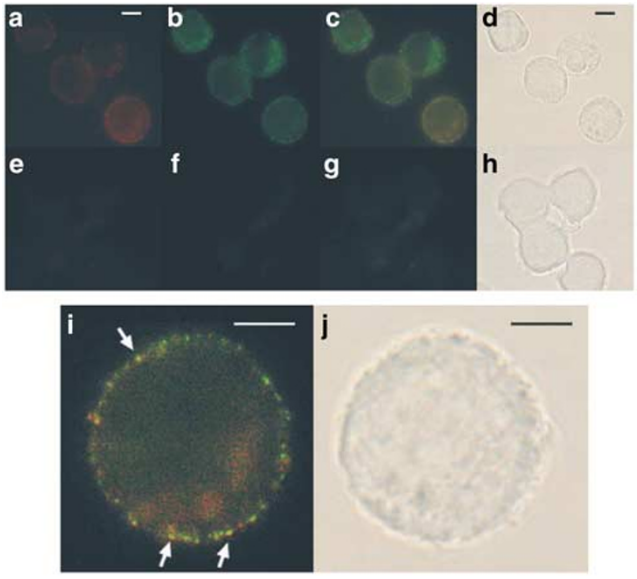


Figure 7 Hsp70/Bag-4 are colocalized on the surface of double-positive tumor cells. CX+ cells were stained at 4°C either with the Bag-4 PE- (**a**), the Hsp70 FITC- (**b**) conjugated or an isotype-matched control antibody in both colors (**e**, **f**). Cells were mounted on slides and analyzed in a fluorescence microscope. Bag-4 positively stained cells are visible in red and Hsp70 positively stained cells in green. Colocalization of Hsp70 and Bag-4 in an overlay is visible as orange spots (**c**). (**i**) A higher magnification of an Hsp70/Bag-4 double-positive stained cell. Corresponding light microscopic views are shown in (**d**), (**h**), and (**j**). Experiments were repeated at least four times and identical results were obtained for Colo+ cells; scale bar, 5 μm

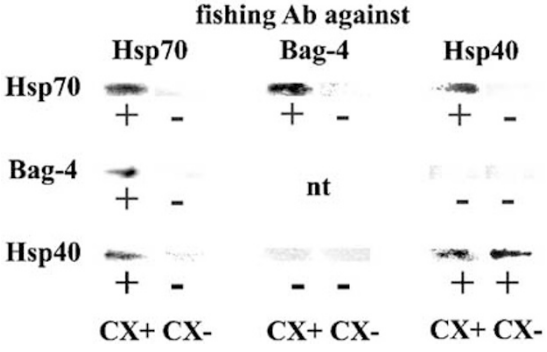


Figure 8 Co-immunoprecipitation of Hsp70, Bag-4, and Hsp40 from purified plasma membrane extracts of CX+/CX− tumored cells. Purified plasma membranes were incubated with beads couple either with Hsp70 (left graph), Bag-4 (middle graph), or Hsp40 (right graph) antibodies. Precipitates were stained with Hsp70 (cmHsp70.1)-, Bag-4 (IMG-152)-, and Hsp40 (SPA-400)-specific antibodies. One representative Western blot analysis of two with identical results is illustrated; results are summarized as '+' and '−' below each graph; nt, not tested

Table 3, a positive cell surface staining was detectable only for Hsp40, but not for other HSPs including Hsp60 and Hsp90. No significant differences in the percentages of Hsp40-positive CX+/CX−, Colo+/Colo−,and HeLa Bag-4/HeLa neo-transfected cells were found (Table 3). Following irradiation, Hsp40 expression was not upregulated significantly (data not shown).

Immunoprecipitation experiments using purified plasma membranes derived from CX+/CX− cells confirmed these findings. As shown in Figure 8 (lower right part), Hsp40 protein could be immunoprecipitated by Hsp40 antibody-coupled beads from plasma membranes prepared from CX+ and CX− tumor cells. As expected, Hsp70 protein could be precipitated selectively from plasma membranes of CX+ tumor sublines but not from that of their Hsp70-negative counterparts (Figure 8, upper part). Interestingly, Hsp70 was found also in precipitations of CX+ plasma membranes, using Bag-4 and Hsp40 antibody-coupled beads. These data indicated that Hsp70 might be associated with Bag-4 and Hsp40 on the plasma membrane of CX+ cells. However, an interaction of Hsp70, Bag-4, and Hsp40 could be excluded due to the fact that Bag-4 and Hsp40 antibody-coupled beads

did not co-immunoprecipitate Hsp40 and Bag-4 together with Hsp70 from CX+ plasma membranes (Figure 8). Similar results were obtained when membranes of Colo+ cells were used (data not shown).

In summary, these data indicated that Hsp70 was associated either with Bag-4 or Hsp40 on the plasma membrane of CX+ and Colo+ tumor sublines. In CX− tumor sublines, Hsp40 was found on the cell surface also in the absence of Hsp70 and Bag-4, as determined by flow cytometry (Table 3) and immunoprecipitation (Figure 8). Other HSPs including Hsp60 and Hsp90 were neither detectable on the cell surface of Hsp70 membrane-positive nor on their negative counterparts (Table 3).

Hsp70/Bag-4 cell surface expression confers protection against γ-irradiation-induced effects

Functional consequences of nonlethal γ-irradiation in tumor sublines with differential Hsp70/Bag-4 and Hsp70/Hsp40 membrane expression pattern were analyzed in a standard [^{3}H]thymidine assay. Irrespective of the treatment, after 12 h, all cells became plastic adherent (data not shown), thus indicating that anoikes was not responsible for the differential cell growth. Representative images of cell cultures photographed after 48 h are shown in the upper part of Figure 9a–c. Growth rates of untreated Hsp70/Bag-4 high-expressing carcinoma cells (CX+, Colo+, HeLa Bag-4) and their low-expressing counterparts (CX−, Colo−, HeLa neo) were comparable, as depicted by light microscopic analysis and by [^{3}H]thymidine uptake (Figure 9a–c). Following irradiation at 1×10 Gy and a 48 h recovery period, proliferation was reduced in all cell types. However, a comparison of irradiated CX+/CX−, Colo+Colo−, and HeLa Bag-4/HeLa neo cells revealed that Hsp70/Bag-4 and Hsp70/Hsp40 high-expressing carcinoma cells appeared to be better protected against irradiation-induced growth reduction, as compared to their low-expressing counterparts. After irradiation at 1×10 Gy, growth reduction was 40% in CX+ cells, 54% in Colo+ cells, and 22% in HeLa Bag-4-transfected cells. In CX−, Colo−, and HeLa neo-transfected cells, growth reduction was significantly elevated: 72% ($^*P<0.00007$, $n=5$), and 69% ($^*P<0.00003$, $n=12$), and 67% ($^*P<0.00007$, $n=8$), respectively.

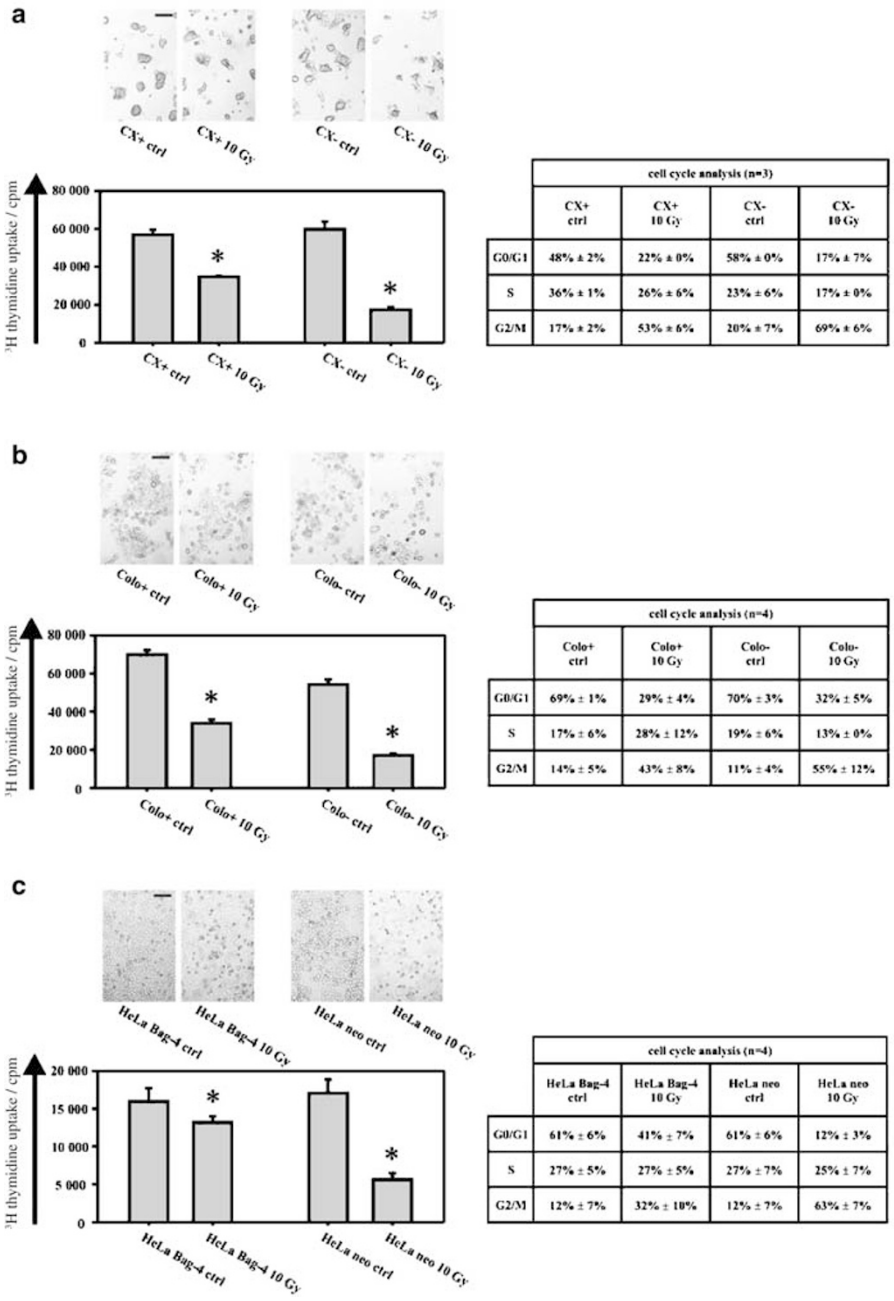
It was known that growth inhibition mediated through irradiation is due to a G2/M cell cycle arrest. Therefore, in addition to the [^{3}H]thymidine uptake assay, cell cycle analyses were performed before and after identical treatment conditions. The typical irradiation-induced G2/M arrest was more pronounced in CX− (G2/M: 69 *versus* 53%, $n=3$), Colo− (G2/M: 55 *versus* 43%, $n=4$), and HeLa new cells (G2/M: 63 *versus* 32%, $n=4$), as compared to their Hsp70/Bag-4 high-expressing partner cell lines. These data further supported the results obtained by [^{3}H]thymidine uptake assays.

γ-Irradiation-induced Hsp70/Bag-4 membrane expression is associated with an increased sensitivity toward activated NK cells

Previously, we demonstrated that membrane-bound Hsp70 provides a target structure for Hsp70 peptide-activated NK cells.[17] Following physical (heat) or chemical (ET-18-OCH3, paclitaxel, vincristinsulfate) stress, the amount of membrane-bound Hsp70 was increased on the cell surface of tumor cells.[19,20,30] Concomitantly, an elevated sensitivity to lysis mediated by Hsp70 peptide-activated NK cells was observed. Here, Hsp70 peptide-activated, CD94 positively enriched NK cells were used as effector cells in cytotoxicity assays and tested against untreated and irradiated tumor cells.[31] As shown in Figure 10, untreated Hsp40 membrane-positive carcinoma sublines with low Hsp70/Bag-4 membrane expression (CX−, Colo−, HeLa neo, filled circles (right part) were lysed less efficiently as compared to their high-expressing counterparts (CX+, Colo+, HeLa Bag-4, filled circles (left part), unless irradiation. After nonlethal γ-irradiation, lysis of Hsp70/Bag-4-positive CX+, Colo+, and HeLa Bag-4 carcinoma cells remained unaltered and high (open circles, left part). In contrast, lysis of irradiated CX−, Colo−, and HeLa neo carcinoma cells, with initially low Hsp70/Bag-4 expression, was significantly enhanced in a dose-dependent manner (open circles, right part). CD3 positively enriched T cells neither showed significant lysis of untreated nor irradiated tumor sublines even after stimulation with Hsp70 peptide (data not shown). Taken together, these data indicated that an irradiation-induced increase in Hsp70/Bag-4 membrane expression (Figures 2 and 5), conferring resistance to γ-irradiation, corresponded to an enhanced sensitivity toward NK cell-mediated cytotoxicity. By incubation of irradiated carcinoma cells with Hsp70-specific antibody cmHsp70.1, a significant inhibition of lysis down to the level of Hsp70/Bag-4 low-expressing tumor cells was detected (filled triangles, right and left parts). In contrast, different antibodies directed against Bag-4 (H-300; IMG-152), Hsp40 (SPA-450; SPA-400), Hsp60 (SPA-806), and Hsp90 (SPA-830) did not affect the cytolytic activity of NK cells (data not shown). These data indicated that although Bag-4 and Hsp40 were present on the cell surface of tumor cells, they are not involved in the recognition mediated by NK cells.

Discussion

Radiation is frequently used in cancer therapy, either as a single regimen or in combination with cytostatic drugs. However, radiation-resistant tumor clones limit the therapeutic efficacy. High cytosolic Hsp70 levels could be correlated with protection against apoptotic cell death induced by stress or exogenous compounds.[4,32] Here, the effects of nonlethal γ-irradiation were analyzed in three independent carcinoma cell systems, with initially divergent Hsp70 membrane expression pattern. Apart from Hsp70, members of the BAG family are known to be involved in the regulation of programmed cell death.[8,32] Six different members of the Bag family could be identified in humans, all sharing a conserved BAG domain and a variable N-terminus. Four of the six members, Bag-1, -3, -4, and -6, interact with the ATPase domain of Hsp70 and Hsc70.[33,34] In addition to Hsp70, BAG proteins also bind to cell surface receptors. Interaction of Bag-1M isoform in concert with Hsp70/Hsc70 to retinoic acid receptors, glucocorticoid, estrogen, and thyroid hormone receptors[33–36] mediates stress signaling pathways that regulate Raf-1/ERK



	cell cycle analysis (n=3)			
	CX+ ctrl	CX+ 10 Gy	CX- ctrl	CX- 10 Gy
G0/G1	48% ± 2%	22% ± 0%	58% ± 0%	17% ± 7%
S	36% ± 1%	26% ± 6%	23% ± 6%	17% ± 0%
G2/M	17% ± 2%	53% ± 6%	20% ± 7%	69% ± 6%

	cell cycle analysis (n=4)			
	Colo+ ctrl	Colo+ 10 Gy	Colo- ctrl	Colo- 10 Gy
G0/G1	69% ± 1%	29% ± 4%	70% ± 3%	32% ± 5%
S	17% ± 6%	28% ± 12%	19% ± 6%	13% ± 0%
G2/M	14% ± 5%	43% ± 8%	11% ± 4%	55% ± 12%

	cell cycle analysis (n=4)			
	HeLa Bag-4 ctrl	HeLa Bag-4 10 Gy	HeLa neo ctrl	HeLa neo 10 Gy
G0/G1	61% ± 6%	41% ± 7%	61% ± 6%	12% ± 3%
S	27% ± 5%	27% ± 5%	27% ± 7%	25% ± 7%
G2/M	12% ± 7%	32% ± 10%	12% ± 7%	63% ± 7%

Figure 9 Hsp70/Bag-4 low-expressing carcinoma sublines are more sensitive to γ-irradiation-induced effects as compared to their high-expressing counterparts. Light microscopic analysis, [^{3}H]thymidine uptake, and cell cycle analysis of either untreated (ctrl) or irradiated (1 × 10 Gy) CX + /CX− (**a**), Colo + /Colo− (**b**), and HeLa Bag-4/HeLa neo (**c**) cells are illustrated. Inhibition of cell growth was significantly (*$P < 0.05$) more pronounced in Hsp70/Bag-4 low-expressing carcinoma cells as compared to their Hsp70-Bag-4 high-expressing counterparts. Results of proliferation assays were repeated 5–12 times. Cell cycle analysis represents mean values of three to four different experiments ± S.E.; scale bar, 200 μm

and cell growth.[37] Bag-3 forms an EGF-regulated ternary complex with phospholipase C-γ and Hsp/70/Hsc70 in human melanoma cells.[38,39] Bag-6, first described in *Xenopus,* interacts with apoptosis-inducing factor *reaper* in *Drosophila* cell.[40] Hsc70 (under physiological conditions) and Hsp70 (following stress) have been found to serve as regulators coupling function of BAG proteins to plasma membrane-localized receptors. We and others determined cell membrane localization of Hsp70 in tumor cells.[17,18] Together with the finding that in the cytosol Hsp70 binds to members of the BAG family, it was conceivable that also on the plasma membrane both molecules are interacting and thus might fulfill regulatory functions in apoptotic cell death.

In the present study, we concentrated on the interaction of Hsp70 and Bag-4, also termed SODD, since other members of the BAG family were not detectable on the cell surface of

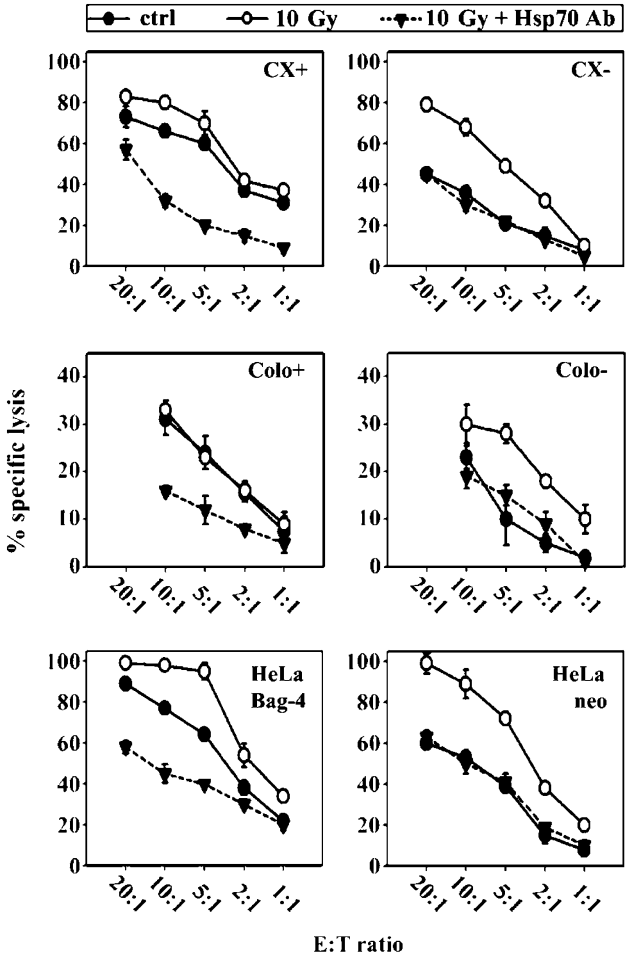


Figure 10 γ-Irradiation-induced increase in Hsp70/Bag-4 on the plasma membrane enhances the sensitivity against the cytolytic attack mediated by Hsp70 peptide-activated NK cells. Cytolytic activity of CD94-enriched, Hsp70-activated NK cells was determined in a CML assay against Hsp70/Bag-4 high- (CX+, HeLa Bag-4, left part) and low-expressing (CX−, Colo−, HeLa neo, right part) carcinoma sublines, either untreated (closed circles) or after γ-irradiation (open circles). Hsp70 specificity was demonstrated by blocking assays using the Hsp70-specific monoclonal antibody cmHsp70.1 (dotted line). Specific lysis was determined at different E : T ratios ranging from 20 : 1 to 1 : 1 for CX+/CX− cells and HeLa Bag-4/HeLa neo cells, and 10 : 1 to 1 : 1 for Colo+/Colo− cells; percentage spontaneous release for each target cell was less than 20%; data represent mean values of two independent experiments

tumor cells. Although our tumor sublines differed significantly in their capacity to present Hsp70 cell surface, no alterations were observed in cytosolic Hsp70 content. Even after nonlethal γ-irradiation, the cytosolic amount of Hsp70 remained unchanged. Comparable results were obtained for cytosolic Bag-4.

In contrast to the cytoplasm, the amount of membrane-bound Hsp70 was significantly upregulated in tumor sublines with initially low Hsp70 cell surface expression following γ-irradiation. Interestingly, the Hsp70 membrane-positive phenotype was associated with a Bag-4 cell surface expression. Other members of the BAG family, including Bag-1 M and Bag1, were not found on the cell surface. Colocalization of Hsp70 and Bag-4 was shown by two-color flow cytometry and immunohistochemistry. These data led us to the hypothesis that Hsp70/Bag-4 protein complexes not only exist in the cytosol but also in the plasma membrane.

We found a positive cell surface staining of Bag-4 only with antibodies reacting with the C-terminus, known to interact with the ATPase domain of Hsp70; antibodies directed against the N-terminus were unable to detect membrane-bound Bag-4. This could be due to the fact that the N-terminal domain of Bag-4 is masked by Hsp70 bound to Bag-4. On the other hand, this might also provide an explanation for the finding that antibodies directed against the ATPase domain of Hsp70 were unable to recognize cell membrane-bound Hsp70.[41]

It has been reported that Hsp70 associated with Bag-4 binds to the death domain of TNFR1 or DR3 and thus prevents self-aggregation to functional trimers initiating caspase-dependent apoptotic cell death.[42] Therefore, we hypothesized that membrane-bound Hsp70/Bag-4 might also be linked to the cytosolic domain of TNFR1. However, neither CX+/CX− nor Colo+/Colo− carcinoma sublines exhibited TNFR1 or DR3 on their cell membranes. Furthermore, incubation of the carcinoma cells with TNF-α either alone or in combination with cycloheximid did not induce apoptosis (data not shown). Regarding these results, we speculated about a TNFR1/DR3-independent cell surface translocation of Hsp70/Bag-4. Hsp70 also plays a pivotal role in cell cycle regulation. Treatment of tumor cells with Hsp70 antisense oligomers resulted in inhibition of proliferation and in initiation of apoptosis in tumor cell lines.[4,43] It has been suggested that overexpression of Hsp70 reduced radiation-induced cell cycle arrest in the G2/M phase. We provide evidence that not only cytosolic but also cell membrane-bound Hsp70/Bag-4 proteins might be involved in protection against irradiation-induced effects. In unstressed cells, the proliferative capacity was comparable in Hsp70 high- and low-expressing tumor sublines. In contrast, following nonlethal irradiation, Hsp70/Bag-4 membrane localization appeared to be more resistant to growth arrest.

Screening for co-chaperones of Hsp70 on the cell surface revealed that Hsp40 was present on the cell surface of all tumor sublines, either in association with Hsp70 or alone. Co-immunoprecipitation studies led us to the hypothesis that in Hsp70 membrane-positive tumor sublines Hsp40 might compete with Bag-4 for binding to the ATPase domain of Hsp70. Regarding these results, we addressed the question as to whether Bag-4 or Hsp40 might be relevant as a target structure for the cytolytic attack mediated by NK cells. Previously, a 14-mer sequence, residing in the C-terminal substrate binding domain of Hsp70, was identified as the extracellular localized part of Hsp70 on tumor cells.[30] Similar to full-length recombinant Hsp70 protein, this peptide was found to stimulate the cytolytic activity of NK cells against Hsp70 membrane-positive tumor cells.[31] Affinity chromatography revealed that selectively Hsp70 protein and Hsp70 peptide facilitated binding and uptake of granzyme B into Hsp70 membrane-positive tumor cells. The mechanism of lysis after contact of NK cells with Hsp70 was identified as granzyme B-mediated apoptosis.[44] These data indicated that only Hsp70 or a peptide derived thereof, but not Bag-4 and Hsp40, provided the target structure for NK cell-mediated

lysis. T lymphocytes neither responded to Hsp70 protein nor to Hsp70 peptide with an increased cytolytic activity.

Recently, several cell surface molecules, including CD40 on B cells,[45] CD14 and toll-like receptors on monocytes and dendritic cells,[46] and CD91 on macrophages,[47] were discussed as potential receptors for HSPs on immunoregulatory cells. The interaction of NK cells with Hsp70 and Hsp70 peptide was found to be mediated through the C-type lectin receptor CD94.[48]

In the present study, we elucidated a dual role for membrane-bound Hsp70, Bag-4, and Hsp40 in radiotherapy. On the one hand, tumor sublines expressing high amounts of Hsp70/Bag-4 and Hsp70/Hsp40 on their cell surface were better protected against γ-irradiation-induced effects, as compared to their low-expressing counterparts, and thus might confer radioresistance. On the other hand, these tumor cells provided ideal targets for the cytolytic attack mediated by Hsp70 peptide-activated NK cells. Blocking studies clearly demonstrated that selectively Hsp70, but neither Bag-4- nor Hsp40-specific antibodies, had the capacity to inhibit NK cell-mediated tumor cell lysis. Together with the finding that the cytolytic capacity of NK cells could be efficiently stimulated by addition of recombinant Hsp70 protein and Hsp70 peptide in the absence of Bag-4 and Hsp40, we concluded that Hsp70 alone is sufficient to sensitize tumor cells for NK cell-mediated lysis.[30,31]

Since an Hsp70 membrane-positive phenotype was frequently found in patient-derived tumor biopsies after *in vivo* irradiation,[49] it is conceivable that these patients might profit from Hsp70-activated NK cells that support the elimination of radiation-resistant tumor cells.

Materials and Methods

Cells and cell culture

The human colon carcinoma sublines CX+ and CX− generated from the parental cell line CX2 (Tumorzellbank, DKFZ Heidelberg, Germany) by cell sorting, using the mouse anti-human Hsp70-specific monoclonal antibody (C-terminus, $aa_{450–463}$, cmHsp70.1, IgG1, multimmune GmbH, Regensburg, Germany), were cultured in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Gibco), 1% antibiotics (penicillin/streptomycin, Gibco), and 2 mM L-glutamine (Gibco). The human pancreas carcinoma sublines Colo+ and Colo− were sorted from the original cell line Colo 357 (CAMR Centre for Applied Microbiology & Research, Salisbury, Wiltshire, UK) following the protocol described for CX+/CX− cells.[29] Growth conditions of pancreas and colon carcinoma sublines were comparable. Exponential cell growth was maintained by regular cell passages. Every 3 days, cells were trypsinized for 1 min with trypsin/EDTA (Gibco) and single-cell suspensions were seeded at a constant cell density of 0.5×10^6 cells in 5 ml medium in T25 ventilated small culture flasks (Greiner, Nuertingen, Germany).

The human cervix carcinoma cell line HeLa was stably transfected either with an expression vector harboring mouse Bag-4 cDNA (pcDNA-SODD, access code Q8CI61, kindly provided by Dr. R Endres, Klinikum MRI, TU-Munich) or with the corresponding control vector (neo). DNA (8 μg) was electroporated into 5×10^6 cells using a BioRad gene pulser (750 V, 25 μF, 200 Ω, 0.4 cm cuvettes), and cells were selected in G418 (0.3 mg/ml, Calbiochem, San Diego, CA, USA) containing medium.[50] Individual subclones stably overexpressing mouse Bag-4 were isolated and cultured, as described above.

Plating efficiency, doubling time, and protein content in Hsp70 high- and low-expressing CX+/CX−, Colo+/Colo−, and HeLa Bag-4/HeLa neo cells were comparable under physiological conditions.

γ-Irradiation

Exponentially growing carcinoma cells were exposed to γ-irradiation, delivered by a ^{137}Cs source at a dose rate of 1 Gy per 10 s, on day 2 following cell passage, either fractionated (2×2 to 5×2 Gy) or with single doses of 1×10 and 1×20 Gy. Cell viability was determined in the adherent and nonadherent cell population by Trypan blue exclusion assays using light microscopy and by Annexin-V–FITC staining on a flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany).

Plasma membrane preparation

Plasma membranes were prepared from 50–100 $\times 10^6$ tumor cells, either untreated or following nonlethal irradiation.[51] After trypsinization, cells were rinsed in 1 ml ice-cold buffer (0.01 M Tris-HCl, 5 mM $MgCl_2$, pH 7.4) and pelleted by centrifugation ($500 \times g$, 4°C). The cell pellets were resuspended in ice-cold Dounce buffer (Tris-HCl, pH 7.6, 0.5 mM $MgCl_2$) and homogenized by 30 strokes in a chilled Dounce homogenizer with a tight-fitting pestle. After adding tonicity restoration buffer (10 mM Tris-HCl, 0.5 mM $MgCl_2$, 0.6 M NaCl, 1 mM PMSF, 1 μg/ml apoprotinin), broken cells were examined in a phase-contrast microscope. After centrifugation ($500 \times g$, 4°C, 5 min), EDTA was added to the supernatant at a final concentration of 5 mM, pH 7.6. After ultracentrifugation for 45 min at $100\,000 \times g$, 4°C, plasma membrane pellets were solubilized in PBS buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1 mM PMSF, 1 μg/ml apoprotinin) by frequent vortexing for 40 min. Insoluble material was discarded by a further centrifugation step at $10\,000 \times g$, at 4°C for 10 min.

Cell lysate preparation, immunoprecipitation, SDS–PAGE, and Western blot analysis

Untreated and irradiated cells were washed twice in ice-cold PBS. After trypsinization and another washing step in PBS, cells were counted and cell pellets (5.0×10^6 cells/ml) were diluted in lysis buffer (120 mM sodium chloride, 40 mM Tris pH 8.0, 0.5% NP-40).

Hsp70, Bag-4, and Hsp40 antibody (5 μg/ml)-conjugated Gamma bind plus protein G–Sepharose beads (Pharmacia) were incubated either with tumor cell lysates or plasma membrane preparations derived thereof for 12 h at 4°C under gentle rotation. After two washing steps, immune complexes were eluted by high salt (NaCl, 3 M) and equal protein amounts (5 μg per lane) were subjected to 10% SDS–PAGE under reducing conditions.[52] After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted according to a protocol of Towbin *et al*.[53] The following antibodies (Table 1), diluted 1 : 1000, were used for Western blot analysis: mouse anti-human Hsp70 (C-terminus, $aa_{450–463}$, cmHsp70.1, multimmune GmbH, Regensburg, Germany), rabbit anti-human Bag-4/SODD (C-terminus, H-300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human Bag-4/SODD (C-terminus, $aa_{443–457}$, IMG-152, Imgenex, San Diego, CA, USA), goat anti-human Bag-4/SODD (N-terminus, N-19; Santa Cruz Biotechnology), goat anti-human Bag-1 (C-terminus; C-16, Santa Cruz Biotechnology), rabbit anti-human Bag-1 (full length; FL-274, Santa Cruz Biotechnology), mouse anti-human Hsp40 (J domain, SPA-450, Stressgen, British Colombia,

Canada), mouse anti-human Hsp60 (C-terminus, SPA-806, Stressgen), mouse anti-human Hsp90 (C-terminus, SPA 830, Stressgen), and mouse anti-human tubulin (clone DM1A, Oncogene, Boston, MA, USA). After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark), also diluted 1 : 1000, protein bands were visualized by chemiluminescence (ECL detection kit, Amersham Biosciences, Little Chalfont, UK). Autoradiographs were recorded on X-omat films (Kodak, Stuttgart, Germany). Prestained standard protein markers (Amersham Biosciences) were used for estimating the molecular weights of each protein.

Flow cytometry

For direct flow cytometry, tumor cells (0.1×10^6 cells per antibody) were counted and incubated either with an FITC-conjugated Hsp70 (cmHsp70.1) or an isotype-matched IgG1 negative control antibody. Following two washing steps and an incubation period of 20 min with 7-amino-actinomycin D (7-AAD, Becton Dickinson Pharmingen, Heidelberg, Germany), viable cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

For indirect flow cytometry, unconjugated antibodies (diluted 1 : 100), directed against Bag-4 (H-300, IMG-152), Bag-1 (FL-274, C-16), Hsp40 (SPA-400), Hsp60 (SPA-806), and Hsp90 (SPA-830), were incubated with tumor cells at 4°C for 20 min. After two washing steps, cells were incubated with the corresponding PE-conjugated secondary antibody at 4°C for another 15 min in the dark and analyzed as described above.

In a double-staining procedure, cells were incubated sequentially with the different antibodies. A detailed description of the different antibodies is summarized in Table 1.

Light and immunofluorescence microscopy

Tumor cells, untreated or irradiated, were stained as described for flow cytometry and transferred to glass slides in Fluorescent Mounting Medium (DAKO, Carpinteria, USA). Slides were analyzed for transmission and fluorescence microscopy on a Zeiss Axioscop 2 Scanning microscope (Zeiss, Jena, Germany) equipped with a $\times 100$ (planar) and a $\times 63$ (apochromatic) oil-immersion objective and standard filters. Multiplicative shading corrections were performed using the software Axiovison (Zeiss Vision, Jena, Germany). Photographs of specifically stained cells sections were taken. The localization of Bag-4 was visualized in red (PE) and of Hsp70 in green (FITC). An overlay of red and green is marked in orange.

[^{3}H]thymidine incorporation assay

Cells were seeded in T-25 culture flasks (Greiner, Nuertingen, Germany) and cultivated for 24 h. Then, exponentially growing adherent cells were irradiated (1×10 Gy), trypsinized, and counted. Appropriate cell numbers (5×10^3 cells/well) were resuspended in culture medium, plated into 96-well flat-bottom plates (150 μl/well), and incubated for another 24 and 48 h in the presence of 1 μCi [^{3}H]thymidine. Irrespective of the pretreatment, 12 h after seeding, all tumor cells became adherent, as determined by phase-contrast light microscopic inspection (data not shown). [^{3}H]thymidine uptake was measured in a scintillation counter (Perkin Elmer Life Sciences, Boston, MA, USA).

Cell cycle analysis

DNA content was analyzed in ethanol (80%)-fixed untreated and irradiated (1×10 Gy) carcinoma cells. Following a washing step in 1 mM EDTA/PBS and resuspension in PBS (1 ml), 10 μl propidium iodide solution (5 mg/ml) and 10 μl ribonuclease (10 μg/ml) were added. Cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) after an incubation period of 20 min at room temperature.

Cell-mediated lympholysis assay (CML) and antibody blocking assays

NK cells were generated from heparinized peripheral blood (200 ml) of healthy human individuals, following Ficoll density gradient centrifugation and CD94 antibody-based magnetic bead cell sorting, according to the CD56 antibody standard protocol (MidiMACS; Miltenyi, Bergisch Gladbach, Germany). After a 4-day stimulation period with low-dose IL-2 (100 IU/ml; Chiron) plus 2 μg/ml Hsp70 peptide TKD ($aa_{450-463}$; TKDNNLLGRFELSG), the cytolytic response was tested against Hsp70 high-expressing CX+, Colo+, and HeLa Bag-4 and low-expressing CX−, Colo−, and HeLa neo cells, either untreated or following irradiation (1×10 Gy) and a recovery period of 24 h, in a standard [^{51}Cr] assay.[54] Briefly, different dilutions of effector and target cells (40 : 1 to 2 : 1) were coincubated in duplicates for 4 h at 37°C, in a final volume of 200 μl. Then, supernatants (30 μl) were collected and radioactivity was measured on a γ-counter (Topcount, Packard instrument). Antibody blocking studies were performed either with Hsp70 (cmHsp70.1), Bag-4 (H-300; IMG-152), Hsp40 (SPA-400, SPA-460), Hsp60 (SPA-806), or Hsp90 (SPA-830) antibodies. Briefly, after radioactive labeling, target cells (0.3×10^6) were incubated with the relevant antibodies (10 μg/ml) for 15 min.

The percentage specific lysis was determined according to the following equation: % lysis = experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Percentage spontaneous release of each target cell was less than 25%.

Miscellaneous

Chemicals were from Sigma (Munich, Germany), Merck (Darmstadt, Germany), or Roth (Karlsruhe, Germany), if not indicated otherwise.

Statistics

For statistical analysis, Student's *t*-test was used. A confidence level above 95% ($P < 0.05$) was determined as significant.

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