Opposite roles of metastasin (S100A4) in two potentially tumoricidal mechanisms involving human lymphocyte protein Tag7 and Hsp70

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Edited by Joseph Schlessinger, Yale University School of Medicine, New Haven, CT, and approved July 8, 2009 (received for review January 6, 2009)

We compare the physical and functional interactions between three widespread multifunctional proteins [metastasin (Mts1/S100A4), innate immunity-related Tag7/PGRP-S, and Hsp70] in two experimental models relevant to host–tumor relationships on humoral and cellular levels. (*i*) Tag7 and Hsp70 in solution or in a lymphocyte make a stable binary complex that is highly cytotoxic for some tumor cells. Here, we show that Mts1 prevents Tag7·Hsp70 assembly in solution, and an excess of Mts1 disrupts the existing Tag7·Hsp70 complex; accordingly, Tag7·Hsp70 cytotoxicity (exemplified with L929 cells) is diminished in the presence of excess Mts1. (*ii*) Tag7 exposed on a specialized subset of lymphokine-activated killer cells makes specific contact with Hsp70 exposed on some HLA-negative tumor cells, thus enabling FasL/Fas-mediated induction of apoptosis. Here, we show that some $CD4^+CD25^+$ cells coexpose Mts1 with Tag7 and FasL, that Mts1 and Tag7 closely contact the same Hsp70 molecule on the target K562 cell (as evidenced by cross-linking), and that killing of such targets is abolished by Mts1-specific antibodies (or selective removal of Mts1-exposing lymphocytes). Thus, this phenotype active against immunoevasive cancerous cells is defined as $CD4^+CD25^+$, $FasL^+$, $Tag7^+Mts1^+$ (≈0.5% of total lymphocytes in culture). Remarkably, similar effectors with at least the same activity are often found in fresh donor blood samples (≈10^4 effectors/mL). Thus, our models suggest that interactions between the three proteins in different situations may have opposite functional outcomes as regards antitumor defense, immune escape, and metastasis.

immune responses to cancer | metastasis

Metastasin (Mts1) belongs to the large S100 family of Ca-binding proteins, and is classified there as S100A4 (1). This small (≈10 kDa) readily dimerizing polypeptide can take part in quite diverse processes both inside and outside the cell (2, 3). Particularly, it has been implicated in tumor metastasis (1, 4–7). However, Mts1 may be highly expressed in various cells of the host defense system (4, 8), and among them, T lymphocytes, which are essential agents of antitumor defense.

We earlier found that in vitro Mts1 could bind to Hsp70 and hinder its interaction with Tag7 (9), a product of the gene identified in this laboratory (10, 11). This evolutionarily conserved ≈20-kDa protein (more broadly known as peptidoglycan recognition protein short form, PGRP-S) is regarded as a component of innate immunity (12–16). Exploring the possible role(s) of Tag7 in antitumor mechanisms, we have shown that (*i*) Tag7 can form a tight and specific 1:1 complex with Hsp70 in vitro; (*ii*) although Tag7 itself is not cytotoxic (to say nothing of Hsp70), the Tag7·Hsp70 complex efficiently induces apoptosis in certain cultured tumor cells and markedly retards tumor growth in mice (9, 17); and (*iii*) the same complex is secreted in bioactive amounts by lymphokine-activated killer cells (LAKs) of the $CD8^+$ type (9).

Studying how LAKs destroy tumor cells, we found another unusual way in which interaction between Tag7 and Hsp70 can contribute to antitumor surveillance. Namely, a 6-day LAK culture, which does not contain natural killer (CD56) cells, is nonetheless capable of killing K562 and MOLT4 cells (18, 19). These cells, derived from hematological tumors, are devoid of the MHC antigen-presenting machinery, and are not recognized by the classical effectors of acquired immunity ($CD8^+$ CTLs). They are also totally insensitive to the soluble Tag7·Hsp70 complex (19). However, there is a specialized subset of $CD4^+CD25^+$ LAKs with surface-exposed Tag7, which binds to Hsp70 exposed on the surface of approximately half of these HLA-negative tumor cells. This interaction is very specific and results in close cell–cell contact that is necessary for subsequent FasL/Fas-mediated induction of apoptosis in the target cell. Notably, $CD4^+CD25^+Tag7^+$ lymphocytes that use the same contact killing mechanism are present in fresh donor blood (19).

The role of such "*in trans*" Tag7–Hsp70 recognition/binding is clearly different from that of the secreted binary Tag7·Hsp70 complex. Also, chemical cross-linking of the proteins closest to each other at the LAK–K562 interface yielded products that contained both Tag7 and Hsp70, but were definitely larger (105–110 and 140 kDa) than the expected Tag7+Hsp70 (90 kDa), and most probably included other lymphocyte proteins (19). We immediately thought of Mts1, because it somehow affected the interaction between Tag7 and Hsp70 in vitro (9), was expressed in T cells (4), and fitted into the minimal size difference between the binary complex and the cross-linked product. Our preliminary tests showed that antibodies against Mts1 decreased the contact-killing capacity of LAKs, although Mts1 itself was devoid of any cytotoxicity.

Therefore, it was reasonable (*i*) to examine in more detail how the interplay of Hsp70, Tag7, and Mts1 can affect the assembly and activity of the soluble complex; and (*ii*) to check whether Mts1 participates along with Tag7 in the novel mechanism active against tumor cells that evade immune surveillance, but expose Hsp70.

Results

Mts1 Interferes with Tag7–Hsp70 Complexing in Solution. To ascertain the ability of Mts1, Tag7, and Hsp70 to interact with each other, the three proteins were tested for coimmunoadsorption on three respective antibody columns. As summarized in Fig. 1*A*

Author contributions: L.P.S. designed research; E.A.D., O.D.K., T.I.L., Y.V.S., D.V.Y., E.A.R., and L.P.S. performed research; E.A.D., Y.V.S., D.V.Y., N.V.G., A.V.G., G.P.G., and L.P.S. analyzed data; and A.V.G. and L.P.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0900116106/DCSupplemental.

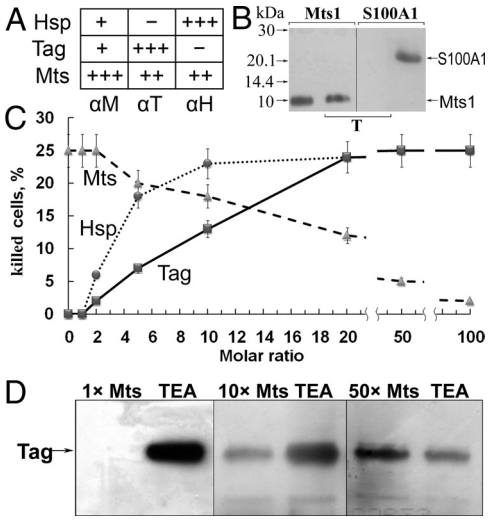

Fig. 1. Competitive relations between Tag7, Hsp70, and Mts1. (*A*) Coimmunoadsorption assay. Aliquots of an equimolar protein mixture (Mts, Tag, Hsp) were passed through columns carrying antibodies to one of the proteins (αM, αT, αH). In the schematic representation, the number of crosses reflects the extent of retention of each protein on each column. (*B*) Specificity of Mts1–Tag7binding. Central gel lanes show the material bound on a column of Sepharose-coupled Tag7 (the T bracket) on loading 10 pmol of either Mts1 (S100A4) or S100A1; outer lanes show the initial proteins (the commercially available S100A1 is a dimer, whereas the A1 and A4 polypeptides are 93/94 residues long). (*C*) Cytotoxic assay with titration. The three proteins were combined in pairs at equal molar concentrations (10 pmol/mL each) in RPMI and incubated for 30 min at 20 °C under sterile conditions. Then, a certain amount of the third protein was added to each pair: (triangles) Mts1 to [Tag7, Hsp70], (circles) Hsp70 to [Tag7, Mts1], or (squares) Tag7 to [Hsp70, Mts1]. The titrant specified at the curve was added in a minimal volume from different stock solutions to achieve the molar ratio (to either protein of the respective pair) indicated on the abscissa axis. The mixture was incubated for another 30 min, and assayed for cytoxicity toward L929 cells as described previously (9). The ordinate gives the percentage (mean ± SEM, $n = 5$) of cells killed by the protein mixture (final dilution 1:10). (*D*) Displacement assay. Biotinylated Tag7 was coincubated with Hsp70 (10 pmol/mL each) for 1 h at 20 °C in 10 mM Tris·HCl, pH 7.5. A 1-mL aliquot was loaded onto a column of Sepharose-coupled antibodies against Hsp70. The column was washed extensively, and then loaded with 1 mL of buffer containing 10, 100, or 500 pmol Mts1. In each case, the eluate was collected (1×, 10×, 50× Mts), and then the material remaining on the column was eluted with 0.25 M triethylamine, pH 12 (TEA). All samples were dried, dissolved in SDS/PAGE sample buffer, run in a 15% gel, blotted onto nitrocellulose, and treated with streptavidin–peroxidase to visualize labeled Tag7 by ECL. The amounts of Tag7 displaced by the increasing amounts of Mts1 (odd lanes from left to right) and retained in complex with Hsp70 (even lanes) can be directly compared throughout the panel as spot intensities. The blots are cropped because there were no other spots; the weak bands seen below may reflect some ''carryover'' of the label (residual BHE) onto Mts1.

(for complete gels and specificity controls, see Fig. S1), each protein in the triple 1:1:1 mixture was completely adsorbed by its antibodies; therewith, the adsorbed Mts1 carried partial amounts of Hsp70 and Tag7, whereas the adsorbed Tag7 carried much Mts1, but no Hsp70, and the adsorbed Hsp70 carried much Mts1, but no Tag7. Under exactly the same conditions, but without Mts1, Hsp70 and Tag7 readily formed the previously described binary complex that was adsorbed both on αT and on αH (Fig. S1*C*). Hence, Mts1 quite efficiently bound with either of the two proteins, but precluded the binding between them.

Interaction of Mts1 with Hsp70 was suggested by our preliminary data (9), and was fully consistent with the general chaperone capacity of Hsp70. The binding of Mts1 with Tag7 was additionally proved by its direct adsorption on an affinity column of immobilized Tag7 (Fig. 1*B*); the figure also shows that the Tag7 column did not bind S100A1, another protein of the same family, confirming the specificity of Mts1–Tag7 interaction.

Table 1. Exposure of Mts1 and Tag7 on LAK cell subsets

Positives (%) among	$CD8^+$	$CD4^+$	$CD4^+25^+$	$CD4^+25^+Mts1^-$
Mts^+	5.0	3.6	11.0	0.6
Tag^+	16.3	10.9	19.8	8.6

Calculated from cell sorter outputs shown in Fig. S2.

Next, we quantitatively examined how the Mts1/Tag7/Hsp70 relationships and the order of their interaction could influence the cytotoxicity in a standard assay (9); it was known that maximally ≈25% of L929 cells could be killed by the binary Tag7·Hsp70 complex at 10^{-10} M or higher. Of course, none of the proteins was toxic if tested alone (up to 10^{-7} M). We prepared two-component equimolar mixtures [Tag7, Hsp70], [Hsp70, Mts1], and [Tag7, Mts1], and incubated them to allow complexing. Then, aliquots of each combination were similarly incubated with increasing amounts of the third component (Mts1, Tag7, or Hsp70, respectively, as indicated at the curves in Fig. 1*C*). All samples were then assayed for cytotoxicity.

Taken by itself, the [Tag7, Hsp70] combination exhibited the maximal expected activity (the upper point on the ordinate axis), whereas [Hsp70, Mts1] and [Tag7, Mts1] were not cytotoxic at all (points at the origin). Addition of an equimolar amount of the third component to any combination did not change anything. However, when the molar ratio of Mts1 to [Tag7, Hsp70] exceeded 2, the cytotoxicity started to decline; half of it remained at a 20-fold excess of Mts1, and practically none at a 100-fold excess. Conversely, on ''titration'' of the inactive pairs, [Tag7, Mts1] with Hsp70, or [Hsp70, Mts1] with Tag7, some cytotoxicity appeared already at a double amount of the titrant, and in both cases, the activity gradually reached that of the Tag7·Hsp70 complex alone; half-maximal effects were attained at approximately a 4-fold excess of Hsp70 and a 10-fold excess of Tag7.

To see what could physically happen in the [Tag7, Hsp70] + Mts1 system, we immobilized the preformed Tag7·Hsp70 complex on an αH column, treated it with Mts1 solutions of increasing concentration, and assayed Tag7 that was thereby released into the eluate, as well as Tag7 that remained Hsp70-bound. As follows from Fig. 1*D*, excess Mts1 indeed displaced Tag7 from the complex in a dose-dependent way, correlating nicely with the cytotoxicity data in Fig. 1*C*.

Mts1 Is Exposed on the Lymphocyte Surface. Pursuing the second goal of the work set in the Introduction, we used the other previously described (19) cellular-level model with LAKs as the source of effectors and erythroblastoid K562 as the source of Hsp70-exposing targets. First of all, we had to check whether Mts1 is present on the surface of lymphocytes along with Tag7. It had already been known (19, 20), and was additionally confirmed here, that K562 were totally devoid of Tag7 or Mts1.

Table 1 summarizes the data of flow cytometry. In the total 6-day LAK culture, both the $CD8^+$ and the $CD4^+$ pools contained a small percentage of $Mts1^+$ cells (one-third of $Tag7^+$ ones). Because it is only the $CD4^+CD25^+$ lymphocytes that use their Tag7 to bind the Hsp70 on the K562 surface (19), we isolated this subset from the total LAKs using antibody-coated magnetic beads. The percentage of $Mts1^+$ among $CD4^+CD25^+$ cells was tripled relative to that in the $CD4^+$ pool, whereas the percentage of $Tag7^+$ cells was doubled, so their ratio approached three-fifths. When $Mts1^+$ cells were removed from the $CD4^+CD25^+$ subset with immunobeads, just as many $Tag7^+$ cells were removed simultaneously (Fig. S2); these data proved that Mts1 was exposed on the same cells as Tag7. Also, microscopic analysis with double immunostaining demonstrated

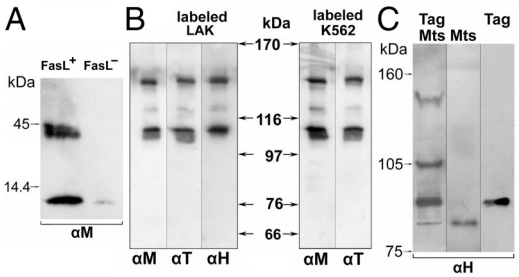

Fig. 2. Assembly of the lymphocyte–tumor cell interface. (*A*) The Mts1-containing material extracted from the surface of FasL$^+$ and FasL$^-$ LAK fractions; size markers are indicated at the left. (*B*) Either LAKs (*Left*) or K562 cells (*Right*) were surface-labeled with biotin and coincubated for 30 min in the presence of 0.2 mM BS^3. The cross-linked material extracted with 1 M KCl was selected on columns with antibodies against Mts1 (αM), Tag7 (αT), or Hsp70 (αH) as specified under the lanes. No labeled material was ever adsorbed on control columns with preimmune rabbit IgG (data not shown). SDS/PAGE was run in 7% gel, size markers are indicated between panels. The gels were blotted onto nitrocellulose; the labeled bands were visualized with streptavidin–peroxidase and an ECL kit. (*C*) Soluble biotinylated Tag7 and Mts1 incubated (together or separately, as indicated above the lanes) with K562 cells and 0.2 mM BS^3; adsorption on anti-Hsp70; 7% gel.

CD4$^+$CD25$^+$ cells carrying both Tag7 and Mts1, as well as those carrying only Tag7 (Fig. S3). The aggregate data show that every ninth or tenth CD4$^+$CD25$^+$ cell was also Tag7$^+$Mts1$^+$.

We additionally checked whether the exposure of Mts1, like that of Tag7 (19), correlated with the exposure of FasL, the apoptosis-triggering agent. FasL$^+$ and FasL$^-$ cells were separated, their surface proteins were biotinylated, solubilized, and the material adsorbed on an anti-Mts1 column was resolved by SDS/PAGE. Practically all labeled Mts1 was in the FasL$^+$ fraction, and only traces in FasL$^-$ (Fig. 2*A*).

Intriguingly, besides the expected Mts1 monomer of ≈10 kDa, there was a no less intense Mts1-containing band ≈40 kDa. This band did not include Tag7 as judged by immunoblotting. A special check revealed that the same ≈40-kDa band developed if a solution of individual Mts1 in PBS was stored for two weeks at 4 °C. Thus, in all probability, the lymphocyte surface (Fig. 2*A Left*) also carried a sturdy Mts1 tetramer.

Mts1 Always Attends Tag7 and Hsp70 at the Cell Interface. To check whether Mts1 is indeed the additional component of the lymphocyte–target cell contact zone, and to get some insight into the spatial arrangement of the latter, we accordingly expanded the five-step procedure used in the preceding work (19). Namely, (*i*) only surface-accessible proteins were biotinylated either on LAKs or on K562, and the cells were carefully washed; (*ii*) K562 and LAKs (only one kind surface-labeled) were briefly coincubated in the presence of a water-soluble cross-linking agent (BS^3, ≈11-Å spacer) under conditions excluding cell lysis, and washed; (*iii*) peripheral membrane proteins were extracted from total cell membranes; (*iv*) the extracts were subjected to parallel immunoadsorptions for Hsp70, Tag7, and Mts1; and (*v*) the material specifically retained on each antibody column was resolved by SDS electrophoresis at different PAG concentrations, and the biotin label (rather than the protein) was visualized on the blots. In this way, the contacts made by each protein on the cell surface during coincubation could be deduced by comparing the label distributions.

With surface-labeled lymphocytes, all three immunoadsorptions yielded exactly the same gel pattern (Fig. 2*B Left*) with two major products: a compact band at ≈140 kDa and a composite band ≈105 kDa (plus a minor band between them). Identical results were obtained when the K562 cell surface was biotinylated, i.e., the target Hsp70 was labeled in the cross-linked complexes (Fig. 2*B Right*).

The label distributions were the same if proteins after cross-linking were extracted directly from whole cells, confirming that there were no labeled products in the cell interior and no cell rupture during coincubation. Thus, we always saw all of the three surface proteins in the same two major bands, and no immunochemically related bands either above or below (for PAGE runs emphasizing the low- and the high-molecular ranges, from 30 to 200 kDa, see Fig. S4). Nothing like these cross-linking patterns could be observed when the entire procedure was applied to LAKs alone (actually, there was no discrete pattern to be shown), to say nothing of K562 cells alone (which contain neither Tag7 nor Mts1).

In additional simulation experiments (Fig. 2*C*), biotinylated proteins (Mts1, Tag7, or their 1:1 combination) were incubated with intact nonlabeled K562 cells and the BS^3 cross-linker, and the extracted Hsp70-containing labeled material was examined by SDS/PAGE. With Tag7 (right lane) there appeared a single 90-kDa band expected for its binary complex with Hsp70; with Mts1 (middle lane), a single band >80 kDa. Remarkably, the [Tag7, Mts1] pair (left lane) gave rise to four bands: 140, 105, 90, and >80 kDa; the upper two bands agreed nicely with the major LAK+K562 cross-linking products (Fig. 2*B*), the lower two exactly corresponded to the Tag7+Hsp70 and Mts1+Hsp70 products, which were not observed on coincubation with whole lymphocytes. From the aggregate data we could conclude that the lymphocyte–tumor cell interface contains Tag7 and Mts1 in close proximity with Hsp70.

Mts1 Is Indispensable for Contact Killing by CD4$^+$ Lymphocytes. Last, we had to ascertain whether surface Mts1 is functionally essential. It was known from the preceding work (19) that the long-established K562 line is heterogeneous in susceptibility to leukocyte effectors. Here, we confirmed that maximally ≈20% of total K562 cells died apoptotically on incubation with CD4$^+$ LAKs, and the process was abolished by antibodies to the four obligate components of the system (Tag7 and FasL on the lymphocyte, Hsp70 and Fas on the target; see Table S1). This percentage agrees with the estimated fraction of Fas$^+$ K562 that expose Hsp70 (19). Thus, the left gray bar in Fig. 3*A* represents almost complete elimination of the susceptible tumor cells.

A different K562 subpopulation (of incidentally the same size) is killed by the CD8$^+$ LAKs, which also use the FasL mechanism, but need neither Tag7 nor Hsp70 (Table S1). Their activity is shown for comparison (white bar in Fig. 3*A*), because both Tag7 and Mts1 were comparably presented on both LAK subsets (Table 1).

In peripheral blood mononuclear cells (PBMCs) freshly isolated from healthy donor blood, we also encountered non-NK activity against HLA-negative tumor cells. It strictly depended on the exposure of CD4, CD25, FasL, and Tag. In contrast, the "usual" MHC-restricted CD8$^+$ CTLs were expectedly ineffective (19). Therefore, we isolated the CD4$^+$ lymphocytes from such ex vivo specimens, and ascertained that they had the same characteristics (Table S1) and could kill at least as many K562 cells as their LAK counterparts did (black bar in Fig. 3*A*).

Addition of anti-Mts1 antibodies (Fig. 3*A Center*) did not affect the activity of CD8$^+$ LAKs, but completely suppressed the activity of CD4$^+$ LAK and ex vivo cells; no effect was produced by antibodies to S100A1, another protein of the same family, or by preimmune IgG (Table S1). Also, the activity of both CD4$^+$ samples was practically abolished on removing the Mts1-exposing cells (Fig. 3*A Right*).

Last, we tested whether exogenous Tag7 and Mts1 could protect the tumor cell against the lymphocyte by occupying the sites required for cell–cell interaction. K562 were preincubated with Tag7, Mts1, or their combination before adding CD4$^+$

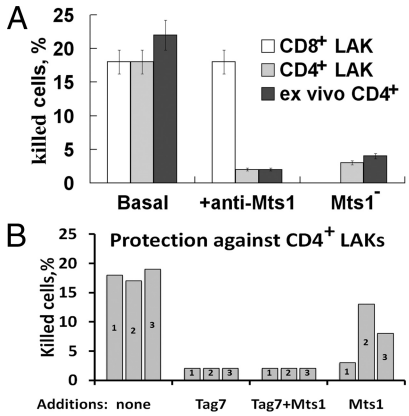

Fig. 3. Necessity of Mts1 for contact killing by $CD4^+$ lymphocytes. (*A*) Basal, activity of the indicated subsets of LAKs and healthy donor PBMC (isolated with immunobeads); +anti-Mts1, the same in the presence of antibodies (20 μg/mL); $Mts1^-$, activity on removal of Mts1-exposing cells with immunobeads (not tested for $CD8^+$). Data are given as mean ± SEM; for LAKs, $n = 5$ (independent cultures); for ex vivo $CD4^+$, $n = 3$ representing selected cases with pronounced Tag7/Hsp70-dependent activity (every fourth blood specimen on average). (*B*) Protection against $CD4^+$ LAKs observed on preincubation of K562 cells with Tag7 and Mts1 (10 nM each). Data are given separately for three independent experiments.

LAKs. The proteins themselves did not affect K562 viability (data not shown). Fig. 3*B* demonstrates that Tag7 itself completely prevented the lymphocyte attack, which was in full accord with the earlier data (19). The [Tag7, Mts1] pair gave the same protection as Tag7 alone. However, Mts1 alone failed to provide reliable protection (three independent experiments without averaging are shown in Fig. 3*B* to compare the variance).

With this exception, the data in Fig. 3 (and the controls in Table S1) actually represent all-or-none effects (complete blocking of lymphocyte activity or no appreciable influence). Thus, Mts1 itself exerts no tumoricidal action, but proves to be an indispensable part of the T cell machinery that can eliminate certain tumor cells.

Discussion

We have examined how a widespread protein Mts1 (S100A4) interacts with two other multifunctional proteins [Tag7 (PGRP-S) and Hsp70] in two systems that model special cases of humoral (9) and cellular (19) antitumor defense. The apparent paradox is that in the former case Mts1 counteracts Tag7·Hsp70 cytotoxicity, i.e., protects the tumor cell, whereas in the latter case, it is an obligate element of contact killing. However, it must be borne in mind that the two mechanisms of inducing apoptosis are certainly different [e.g., L929 cells are devoid of the Fas receptor (9)] and most importantly, Mts1 in these two cases acts at different levels.

In the first model, soluble Mts1, Hsp70, and Tag7 make all three possible pairs (Fig. 1*A*; Fig. S1), but we see no compelling evidence for a stable ternary complex. Anyway, the only cytotoxic form is the binary Tag7·Hsp70, and the changes in activity during reciprocal titration (Fig. 1*C*) reflect competitive displacement. We also directly demonstrate disruption of the preassembled Tag7·Hsp70 complex by excess Mts1 (Fig. 1*D*). Thus, elevated amounts of Mts1 around a tumor cell can simply inactivate the approaching cytotoxic agent. This effect alone would reduce the observed killing rate, and so far, we have no grounds for suggesting any other mode of Mts1 action in this system.

Surveying the toxicity of recombinant or natural (lymphocyte-secreted) Tag7·Hsp70 and of TNFα for various tumor cell lines, we have found that their efficacy negatively correlates with the level of Mts1 expression in the target cells. A vivid example is provided by two murine adenocarcinoma cell lines: nonmetastatic CSML-0, which lack Mts1, are very susceptible, whereas the highly metastatic CSML-100, which overexpress Mts1, are absolutely resistant to Tag7·Hsp70 and TNFα (21). Again, it is known that S100A4 (Mts1) is often elevated in the human breast cancer interstitial fluid (2), originating from stromal, as well as from tumor cells. Thus, we can say that our simple model represents a physiologically plausible situation, be it a solid tumor or a solitary Mts1-high cell in the circulation. Without prejudice to the already known functions of this versatile protein, our data can add a new angle to its involvement in tumor progression and metastasis.

In the second, cell–cell model, the interaction between Tag7 and Hsp70 is no less strong and specific than in solution, but, as outlined in the Introduction, it serves a totally different purpose [target recognition and anchoring (19)], and has no cytotoxic function of its own: it is enough to recall that binding of soluble Tag7 to Hsp70 on K562 cells (Fig. 2*C*) not only does not kill them, but on the contrary, fully prevents the action of the $CD4^+$ LAKs (Fig. 3*B*). Here, we show that Mts1 invariably accompanies Tag7 and Hsp70 at the lymphocyte–tumor cell interface. Most importantly, there are no "partial" cross-linked products of any size that contain only one or two of these three proteins. Such products would have been immediately seen as additional or missing bands on comparing the gels from different antibody columns. The smallest apparent size of the cross-linked products (Fig. 2*B*; Fig. S4) approximately equals the sum of the three polypeptides, and the existence of two Mts1 forms on the lymphocyte (Fig. 2*A*) largely accounts for the presence of 105- and 140-kDa bands throughout these experiments. Anyway, it is certain that each Tag7 molecule together with an "Mts1 unit" make contacts with one and the same target Hsp70 molecule. This issue means that a definite organization (rather than mere presence) of these proteins on the lymphocyte surface is essential to proper cell–cell binding.

The particular place and function of Mts1 in the tumoricidal machinery remain to be established. Most simply, Mts1 may help the surface Tag7 to assume the required conformation and orientation, or more likely, assist in cooperative interactions to secure the lymphocyte on the target. Multipoint binding is required by general considerations and is suggested, among other things, by confocal micrographs (19), showing that in the zone of lymphocyte–tumor cell contact a group of Tag7 sites encircles a single Fas site. Still more interestingly, Mts1 may take part in functional coupling between the recognition step (Tag7/Hsp70) and the death-inducing step (FasL/Fas). The involvement of intracellular and extracellular S100A4 in various signal pathways is broadly discussed in the literature (refs. 1–3 and references therein), but there are no data for its location, state, or interactions in the cell membrane. Our experiments with BS^3 simply could not capture any contacts between proteins under the cell surface. The putative roles of Mts1 are not mutually exclusive; this question is a challenge for further study.

In one way or another, Mts1 is absolutely required for the contact killing function. The virtually identical results obtained with $CD4^+$ LAKs and with fresh donor lymphocytes (Fig. 3*A*; Table S1) prove that this novel mechanism is not an artifact of cell culture, but is physiologically relevant. The share of these $CD4^+CD25^+$, $FasL^+$, $Tag7^+Mts1^+$ cells among our LAK lymphocytes can now be estimated at ≈0.5% as the product of rounded fractions of cells with each additional surface marker: 1/2 for $CD4^+$ × 1/10 for $CD25^+$ × 1/6 for $FasL^+Tag7^+$ (19) × 3/5 for $Mts1^+$ (Table 1) = 1/200. Because normal PBMC also contain several percentage of $CD4^+CD25^+$ cells (22, 23), the above estimate may approximately apply in vivo. Also, in our assays the total ex vivo samples (19) or their $CD4^+$ subsets (Fig. 3*A*) were at least as active as $CD4^+$ LAKs, so they perhaps

contained approximately the same number of these specialized Hsp70-seeking effectors. Thus, the LAK model reflects the naturally occurring situation even in the quantitative aspect.

The modest 0.5% in fact means ≈10,000 of such effectors in every milliliter of healthy blood, capable of killing at least as many tumor cells that represent a radical mode of immune escape (missing self). These effectors can diminish the chances of survival for solitary metastatic cells in the circulation.

Summing up, there is actually no contradiction in that the interplay of Tag7, Hsp70, and Mts1 in essentially different circumstances can have functionally opposite outcomes at a higher (physiological) level, but there are quite a few outstanding questions that remain unstudied.

Materials and Methods

Cell Cultivation and Fractionation. Transformed murine fibroblast L929 and human erythroblastoid K562 cells were cultured in RPMI MEDIUM 1640 with 2 mM L-glutamine and 10% bovine calf serum (Invitrogen). PBMC were isolated from samples provided by official donor stations and either incubated with IL2 (17, 19) for the LAK assays, or used directly for ex vivo assays. Lymphocyte subsets were obtained with commercially available magnetic bead isolation kits (Dynal Biotech ASA) by the manufacturer's protocols; also, rabbit antibodies against Tag7 or Mts1 (S100A4) were attached to beads coated with anti-rabbit IgG.

Proteins and Antibodies. Tag7, Hsp70, as well as rabbit anti-Tag7 and anti-Hsp70 used in immunoadsorption were obtained as previously (9); Mts1 was isolated and purified as described elsewhere (24); S100A1 was from Alpha Diagnostics. Rabbit polyclonal anti-Mts1 (S100A4, Ab-8) were from Neo Markers; rabbit polyclonal anti-S100A1 from Alpha Diagnostics. Monoclonal anti-PGRP-S was from Imgenex, polyclonal anti-FasL from Santa Cruz Biotechnology. R-phycoerythrin-labeled antibodies to CD4, CD8, and CD25 were from Caltag Laboratories; FITC- and Cy3-labeled secondary antibodies were from Sigma.

Biotinylation and Cross-Linking. Soluble proteins were labeled with biotin N-hydroxysuccinimide ester (BHE) (Pierce) at a 1:100 molar ratio for 2 h at 20 °C and dialyzed against PBS for 18 h at 4 °C. The cell procedures were based on the published protocol (25). LAK and K562 cells were surface-labeled with BHE as in the preceding work (19), coincubated, and cross-linked with bis(sulfosuccinimidyl)suberate (BS^3; Pierce) for 30 min at 4 °C. Biotinylated Tag7 and Mts1 were incubated (10 nM each) with K562 ($5{\cdot}10^7$ cells in 1 mL of PBS with 50 mM Hepes, pH 8.3) and 0.2 mM BS^3 for 30 min at 4 °C. After cross-linking, the cells were washed twice in PBS, total membranes were isolated (26), and the peripheral proteins were solubilized with 1 M KCl.

Immunoadsorption and PAGE. Rabbit polyclonal antibodies affinity-purified on the corresponding antigens (9) and preimmune rabbit IgG (as control) were coupled to cyanogen bromide-activated Sepharose by the standard protocol. Loaded columns were washed with PBS, then with PBS plus 0.5 M NaCl, and eluted with 0.25 M triethylamine, pH 12. SDS/PAGE was conducted as previously (9), and biotinylated products were visualized on nitrocellulose blots with horseradish peroxidase-conjugated streptavidin and an ECL Plus kit (all from GE Healthcare).

Contact Killing. K562 cells were cultured in 96-well plates at a density of 5×10^3 cells per well, lymphocytes (20:1) were added in 100 μL. In 3 h at 37 °C, cell death was determined with a Cytotox 96 Assay kit (Promega), always subtracting the values corresponding to spontaneous cell death (within 3%). In every series, the instrumental measurements were independently verified by expert microscopic analysis with Trypan Blue (at 6-fold higher cell densities); the results always agreed within the SE.

Flow Cytometry. The cells were stained with appropriate antibodies and fixed with 1% paraformaldehyde (Sigma) at 20 °C. The samples (at least 10^4 cells each) were analyzed with a Cytomics FC500MPL instrument (Beckman Coulter). The data were processed with CXP Software (version 2.2; Beckman Coulter).

ACKNOWLEDGMENTS. This work was supported by the Russian Foundation for Basic Research, the Russian Academy of Sciences program for Molecular and Cellular Biology, and the International Centre for Genetic Engineering and Biotechnology.

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