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Changes in the Balance between Membrane-Bound and Soluble Forms of CD95 (Fas) during Selection of Tumor Cells *in vivo*

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Abstract—Studies concerning the expression of the receptor CD95 (Fas) by tumor cells and the role of this protein in apoptosis induced by the effector host cells that bear Fas-ligand are mainly focused on the membrane-bound form of Fas. There are only a few data about the production of the soluble form of Fas by the tumor cells, its role in the interaction with the effector host cells, and the possible changes in the synthesis of this protein during tumor progression. In the present work, three *in vitro* transformed parental cell lines of different origin and 24 of their variants isolated after a short cycle of natural selection *in vivo* were studied. It was demonstrated for the first time that: 1) production of the soluble Fas by all selected *in vivo* variant tumor cell lines increased significantly (2-10-fold) in comparison to the initial (parental) cell lines and did not depend on the origin of the parental lines. At the same time, the expression of the membrane-bound form of Fas decreased considerably; 2) variations of the balance between membrane-bound and soluble forms of Fas in selected *in vivo* variant cells and the expression of the $[H_2O_2{}^{CA} + PGE^S]$-phenotype by these cells (this phenotype determines one of the essential mechanisms of the protection of a tumor cell *in vivo*) possibly represent independent secondary changes acquired during tumor progression *in vivo*.

Key words: CD95 (Fas), membrane-bound and soluble isoforms, selection of tumor cells *in vivo*, bcl-2, Rous sarcoma virus

Death or survival and subsequent progression of tumor cells (TC) *in vivo* are determined to a considerable extent by the balance between the efficiency of the mechanisms of TC protection from the effectors of natural immunity and the efficiency of TC elimination by these effectors. Elimination of TC is caused by cytotoxic activity (CTA) of macrophages, neutrophils, natural killers (NK), and T-lymphocytes and is mediated by various mechanisms of recognition and induction of apoptosis of TC. Fas-ligand (FasL) is one of the factors that induce apoptosis in a cell. FasL is expressed by activated NK cells, macrophages, and cytotoxic T-lymphocytes [1]. Membrane-bound receptor of this ligand, mFas, transmits the apoptotic signal to a cell through the caspase cascade [2]. The existence of several soluble splicing forms (sFas) was demonstrated for Fas, with one dominating form (FasTMdel) bearing a deletion of the transmembrane domain. All other forms are present in extremely small quantities [3-5]. The data about the involvement of Fas–FasL interactions both in host protection from TC and in protection of TC themselves from the effector cells of the host immunity including the immunoprivileged zones are presented in a number of recent experimental studies and reviews [2, 6, 7]. However, the data in the literature concerning this question are contradictory, in particular, both the role of FasL in the immunoprivileged zones of the host organism and the possible variations in the expression of different Fas isoforms and in the balance between them during tumor progression are unclear [8].

There is only a single report about possible correlation between mFas and sFas synthesis in TC, as well as about their changes during tumor progression. It was demonstrated recently that the expression of mFas decreases in NIH3T3 line TC during their selection *in*

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vivo, but the quantity of sFas secreted by the cells was not measured in this study [9].

While mFas serves as a target on TC for the effector host cells bearing FasL, the soluble form of the protein may possibly bind (neutralize) FasL on activated NK cells, T-lymphocytes, and macrophages, thus preventing distantly the CTA of these cells directed towards TC. There are very few data concerning sFas production by TC, possible variations of its synthesis during tumor progression, and its probable role in TC protection from the effectors of the host immunity [10]. A few publications indicating the possibility of FasL production by TC and, in this case, for the ability of TC to kill T-lymphocytes remain questionable [8, 11].

Certain other mechanisms of TC protection from the effectors of the natural immunity system are acquired during *in vivo* selection: increase of TC resistance against active forms of oxygen generated by macrophages and neutrophils (in particular, H_2O_2-catabolyzing activity ($H_2O_2^{CA}$)), and also the ability of TC to secrete prostaglandin E_2 during contact with NK cells leading to the inactivation of CTA of the latter. It was demonstrated that these two biochemically different characteristics of TC are coexpressed as a cluster of properties united in the [$H_2O_2^{CA}$ + PGE^S]-phenotype [12-14]. It seemed interesting to elucidate if there is any relationship between two forms of TC protection—expression of different Fas isoforms and the [$H_2O_2^{CA}$ + PGE^S]-phenotype.

Since we possess a collection of cell lines of diverse origin at different stages of tumor progression that are characterized by certain parameters including the expression of the [$H_2O_2^{CA}$ + PGE^S]-phenotype, and a test-system for the quantitative measurement of the membrane-bound and soluble Fas, we devoted this study to the investigation of the following three questions. 1. Does the natural selection of TC *in vivo* influence the level of mFas and sFas expression? 2. Is there any relationship between sFas production and the expression of mFas in the cells of individual TC lines? 3. Is there a correlation between the acquiring of the [$H_2O_2^{CA}$ + PGE^S]-phenotype by TC during *in vivo* selection and the production of different Fas isoforms?

MATERIALS AND METHODS

Cells. The following three cell lines of *in vitro* transformed Syrian hamster fibroblasts were used as parental cells in this study (these cell lines were not subjected to selection *in vivo* before and initially differed by the origin and expression of the [$H_2O_2^{CA}$ + PGE^S]-phenotype): 1) spontaneously transformed (line STHE); 2) cells of STHE line transduced with bcl-2 gene (line STHE-bcl-2); 3) transformed with Rous sarcoma virus (strain Schmidt–Ruppin) (line HET-SR). It was demonstrated earlier that the cells of STHE and STHE-bcl-2 lines, in contrast to HET-SR, initially did not express the [$H_2O_2^{CA}$ + PGE^S]-phenotype, but during their growth *in vivo* natural selection of variants occurred that expressed this phenotype. In conditions of local (subcutaneous) growth the rate of the selection of variant STHE-bcl-2 lines was significantly reduced in comparison to that of variant STHE lines [12, 13].

TC were selected *in vivo* according to the method described earlier [11] as follows: parental transformed *in vitro* cells at dose $2.0 \cdot 10^6$ in 1 ml were injected intravenously into the *sinus retroorbitalis* of Syrian hamsters (model of artificial hematogenic dissemination), followed by sacrifice of the animals using ether after different periods (5, 10, 15, and 20 days) and sterile isolation of the lungs (at these time points without macroscopic changes). The lung tissue of individual animals was cut into small tissue fragments (1-2 mm) and placed into cell culture medium RPMI-1640 or F-12 containing 10% fetal calf serum and the mixture of antibiotics (gentamicin (100 U/ml), amphotericin B (100 U/ml)). *In vitro* TC gradually released from fragments of lung tissue that floated in the medium, accumulated on the bottom of the culture flasks and began to propagate. The non-propagating cells and the fragments of the lung tissue were eliminated gradually (during changes of the medium). The variant TC lines obtained from individual animals in this way under the conditions of the artificial hematogenic dissemination were designated as LO-lines with numbers. In all, 24 variant lines were studied in this work (besides the parental cell lines). These variant lines differed in the period of *in vivo* selection and expression of the [$H_2O_2^{CA}$ + PGE^S]-phenotype.

Measurement of sFas production. An ELISA-sandwich test system was used in this study in order to detect quantitatively the soluble Fas in human blood serum and other biological fluids [15]. The full-length baculovirus Fas (Coultronics, France) was used as a positive control. The lower limit of sensitivity of the test system was 0.6 ng/ml of the full-length Fas.

The monoclonal antibodies SA-7 and SA-8 that were used for the elaboration of the test system could be also applied for the Western-blot analysis against both the full-length Fas and an extracellular fragment of human and hamster Fas (data not shown).

Preparation of culture medium samples for measurement of soluble Fas production by the examined cell lines. Monolayer cultures of the cells were taken off the glass using Versen solution, and counted in Goriayev chamber. An inoculum of $1.0 \cdot 10^7$ cells was plated in Karrel flasks in medium containing 5% embryonic calf serum. After monolayer formation (within 24 h), the cells were washed three times with Hanks' solution to remove the medium with serum and were incubated for 48 h in the RPMI-1640 medium free of serum. After 48 h the medium from the flasks was collected, centrifuged at 120*g* for 5 min, and standardized for protein content by the Bradford

method. Afterwards the content of sFas in the medium was measured by the ELISA-sandwich method. After the removal of the medium from the flasks, the cells producing Fas were taken off the glass using Versen solution. The total cell number and the percent of living cells was assessed in the Goriayev chamber using 0.4% solution of trypan blue. In the cases when the total cell number in the flasks decreased significantly (>5%), and the percent of living cell was lower than 95%, the supernatant was not taken in the study. The culture medium samples were stored at −20°C before use.

Immunoenzymatic assay. Monoclonal antibodies against Fas SA-8 (IgG_1 ($\kappa \approx (4 \pm 0.6)\cdot 10^7$)) were absorbed on plates for the immunoenzyme assay (Linbro) in 0.05 M carbonate buffer, pH 9.6, at the concentration of 5 μg/ml overnight at 4°C.

To inactivate the residual free binding centers the plates were incubated with 1% solution of bovine serum albumin (BSA) in phosphate buffered saline, pH 7.2, for 1 h at 37°C. The supernatants were added to the plates afterwards. The positive control was obtained by addition in each plate of serial twofold dilutions of the recombinant full-length Fas at concentrations from 20 to 0.15 ng/ml. The plates were incubated 2 h at 37°C.

After incubation, the plates were intensively washed with PBS containing 0.1% of Tween 20 (Sigma, USA) (washing buffer). The washing procedure was then repeated after every stage of the test.

After washing, the plates were overlaid with biotinylated monoclonal antibodies against Fas SA-7 (IgG_1 ($\kappa \approx (5.8 \pm 0.7)\cdot 10^8$)) at concentration of 12 μg/ml in PBS containing 0.1% Tween 20 and 0.1% BSA. The plates with biotinylated antibodies were incubated 3 h at 37°C.

In the following step a solution of streptavidin-peroxidase (Amersham) in the working dilution was added to the plates in the washing buffer. The plates were incubated 1 h at 37°C.

Solution (0.04%) of *o*-phenylenediamine (Sigma) in 50 mM citrate-phosphate buffer, pH 5.0, containing 0.03% of hydrogen peroxide was added to the plates. The plates were incubated 15-20 min at room temperature until the development of coloration. The reaction was stopped with 10% sulfuric acid, and the optical density was measured at 492 nm on an MR 700 Microplate Reader (Dynatech Labs, USA).

The concentration of sFas was assessed using the calibrating curve corresponding to each plate.

Detection of mFas expression. mFas expression was detected by indirect immunoenzyme assay using SA-8 monoclonal antibodies; the attached living TC were used as an antigen [16]. For the performance of the assay TC were plated in 96-well cell culture plates ($1\cdot 10^5$ cells per well) (Corning Costar and Linbro Titertek) in RPMI-1640 medium containing 5% fetal calf serum and antibiotics. Within 3 h after seeding, the cells formed monolayers in the wells and were then studied for mFas expression. After incubation, the plates were washed three times with PBS (washing buffer). The washing procedure was repeated after each step of the test. The cells in the wells were then overlaid with SA-8 monoclonal antibodies against Fas (IgG_1 ($\kappa \approx (5.8 \pm 0.7)\cdot 10^8$)) and incubated 2 h at 37°C.

In the following step the cells were overlaid with anti-mouse antibodies conjugated with horseradish peroxidase (Amersham, Holland) at working concentration in PBS containing 0.1% BSA, and incubated 1 h at 37°C.

Then a solution of 0.04% *o*-phenylenediamine in 50 mM citrate-phosphate buffer, pH 5.0, containing 0.03% hydrogen peroxide was added to the plates. The plates were incubated 15-20 min at room temperature until coloration developed. The reaction was stopped with 10% sulfuric acid, the optical density was measured at 492 nm on the MR 700 Microplate Reader. The cell viability and their quantity during the detection procedure were monitored with 0.4% solution of trypan blue in a parallel set of experiments.

The [$H_2O_2^{CA}$ + PGE^S]-phenotype was defined according to the methods described in [12-14] that include the detection of the antioxidative activity of TC and their ability to release PGE_2. H_2O_2 catabolizing activity ($H_2O_2^{CA}$) was measured using luminol-dependent chemiluminescence (LDC) of prepared *ex tempore* extracts of the TC. The cells were extracted in phosphate buffer at pH 7.0, lysed in Triton X-100 at 4°C, centrifuged (8000*g*, 15 min, 4°C), and then the protein content was determined according to Bradford. One hundred microliters of 10 mM H_2O_2 and 0.1 ml of luminol solution (0.01 M, Serva, Germany) were added to 0.1 ml of TC extract that contained 2.0 mg of protein per ml and were immediately placed in the thermostatted (37°C) chemiluminometer (Biolumate, Model 9500, Berthold, Gossheim, Germany). LDC of the samples of the studied cellular extracts and control samples was recorded every 10 sec in the dark until the complete degradation of H_2O_2. $H_2O_2^{CA}$ of TC was considered as positive when the period of 95% degradation of 10 mM H_2O_2 added to the extract varied between 10 and 180 sec. TC characterized by low levels of $H_2O_2^{CA}$ generally catabolized 95% of 10 mM H_2O_2 within 5-20 min and more. Degradation of 95% of 10 mM H_2O_2 added to phosphate buffer normally took 20 min.

For the detection of PGE^S-activity of TC, a sensitive biological test based on the ability of PGE_2 to inactivate CTA of NK cells was elaborated. This method allows the detection of biological activity of the native PGE_2 that is secreted by TC and possesses several important advantages over radioimmunoassay [12, 13, 17].

RESULTS AND DISCUSSION

In the first three sets of experiments (Tables 1-3), we measured sFas production by both initial parental cell

Table 1. sFas production and expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype by STHE cells and their *in vivo* selected variant lines

Cell line	Period of *in vivo* selection, days	sFas production, (pg/cell/48 h) $\times 10^{-3}$	Expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype
STHE	0	0.1 ± 0.03	—
STHE-LO-1	5	0.7 ± 0.03	—
STHE-LO-2		0.97 ± 0.05	+
STHE-LO-3		0.59 ± 0.03	—
STHE-LO-4		0.81 ± 0.01	—
STHE-LO-5		0.68 ± 0.04	—
STHE-LO-6	10	0.98 ± 0.05	+
STHE-LO-8		1.0 ± 0.1	+
STHE-LO-9		0.81 ± 0.03	—
STHE-LO-11	15	0.93 ± 0.04	+
STHE-LO-12		0.98 ± 0.05	+
STHE-LO-13		0.69 ± 0.01	—
STHE-LO-14		0.99 ± 0.05	+
STHE-LO-16		>1.0	+

lines that were transformed *in vitro* and variant TC lines that were subjected to *in vivo* selection for 5-20 days in the conditions of artificial dissemination. The results were compared with the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype by the studied cell line variants.

As can be seen from the data presented in Tables 1 and 2, all variant lines of STHE and STHE-bcl-2 cells without any exception produced significantly higher quantities of soluble Fas in comparison to the corresponding initial parental lines already after 5 days of *in vivo* selection. Since the cells of parental STHE-bcl-2 line initially produced slightly greater quantities of sFas than STHE cells, the relative increase of sFas production by variant STHE-bcl-2 cells was approximately 1.5-2-fold, while sFas production in STHE TC lines increased more than 7-10-fold. The absolute values of sFas production by the cells of variant STHE and STHE-bcl-2 lines coincided completely. Comparison of these data with the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype during *in vivo* selection of the same variant TC lines showed a significant dependence of the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype on the period of *in vivo* selection. Thus, one out of five studied variants of STHE TC expressed the $[H_2O_2^{CA} + PGE^S]$-phenotype after 5 days of *in vivo* selection, while after 10 and 15 days of TC selection *in vivo* already six out of eight of these variants expressed the $[H_2O_2^{CA} + PGE^S]$-phenotype. Comparison of the rates of sFas production and expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype by individual variants of STHE TC (Table 1) demonstrated that in all seven cases a significant increase of sFas production (9-10-fold and more) correlated with the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype; a lower (6-8-fold) increase of sFas production, particularly at early periods of TC selection, preceded the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype. These data provided evidence that both the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype and the increase of sFas production by variant TC lines were caused by TC selection *in vivo*.

In contrast to variants of the STHE line, not a single line from seven variant STHE-bcl-2 TC lines acquired the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype during 5-20 days of *in vivo* selection. Within this period a significant increase of sFas production by TC of all these lines was retained on the same relative level. Our earlier studies demonstrated for the first time that during local (subcutaneous) growth of STHE-bcl-2 cells *in vivo* a considerable delay of the selection of malignant TC variants expressing

Table 2. sFas production and expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype by STHE-bcl-2 cells and their *in vivo* selected variant lines

Cell line	Period of *in vivo* selection, days	sFas production, (pg/cell/48 h) $\times 10^{-3}$	Expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype
STHE	0	0.1 ± 0.03	—
STHE-bcl-2	0	0.44 ± 0.03	—
bcl-2-LO-1	5	0.93 ± 0.04	—
bcl-2-LO-6	10	0.98 ± 0.05	—
bcl-2-LO-9	15	0.7 ± 0.04	—
bcl-2-LO-14	20	0.98 ± 0.05	—
bcl-2-LO-16		0.88 ± 0.04	—
bcl-2-LO-18		0.93 ± 0.04	—
bcl-2-LO-19		0.72 ± 0.03	—

Table 3. sFas production and expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype by HET-SR cells and their selected *in vivo* variant lines

Cell line	Period of *in vivo* selection, days	sFas production, (pg/cell/48 h) $\times 10^{-3}$	Expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype
STHE	0	0.1 ± 0.03	—
HET-SR	0	0.37 ± 0.03	+
HET-SR-LO-1	4	>1.4	+
HET-SR-LO-2		>1.4	+
HET-SR-LO-3		>1.4	+
HET-SR-LO-4		>1.4	+

Table 4. mFas quantity in the cells of different parental and variant lines that differ in their origin, period of *in vivo* selection, and expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype

Cell line	Period of *in vivo* selection, days	mFas, relative units	Expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype
STHE	0	7.2	—
STHE-LO-4	5	2.7	—
STHE-LO-13	15	3.1	—
STHE-LO-14		2.15	+
STHE-LO-16		2.1	+
STHE-bcl-2	0	6.4	—
bcl-2-LO-1	5	1.47	—
bcl-2-LO-6	10	1.39	—
bcl-2-LO-14	20	1.37	—
HET-SR	0	4.65	+
HET-SR-LO-4	4	0.71	+

the $[H_2O_2^{CA} + PGE^S]$-phenotype occurred. Apparently, this delay was caused by the apoptotic activity of the bcl-2 gene product that favored the survival *in vivo* of a greater number of initial cells and thus prevented the appearance and/or selection of their rare variants expressing the $[H_2O_2^{CA} + PGE^S]$-phenotype [13]. It was demonstrated in the studies of other investigators that apoptosis induced by Fas-ligand in TC expressing Fas could not always be blocked by bcl-2 [18]. However, our earlier published data provided evidence that the antiapoptotic activity of the bcl-2 gene product is strongly manifested in the cells of the STHE-bcl-2 line during their local growth and selection *in vivo* [13]. This study confirmed the delay of *in vivo* selection of STHE-bcl-2 cells by the $[H_2O_2^{CA} + PGE^S]$-phenotype also on the model of artificial dissemination of TC. However, as can be seen from the data presented, the delay (or suppression) of the selection of variant lines of STHE-bcl-2 TC by expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype was not hindered by the increase of sFas production by these cells.

Thus, analysis of the results presented in Tables 1 and 2 suggested that the compared properties of TC—the increase of sFas production and the expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype by variant STHE and STHE-bcl-2 TC lines—were acquired independently. The data from the study of the third parental line, HET-SR, did not contradict this conclusion. The variants of HET-SR line initially had a relatively low rate of sFas production, but expressed the $[H_2O_2^{CA} + PGE^S]$-phenotype without *in vivo* selection. The HET-SR variant cells, which were isolated from the lung tissue four days after the injection *in vivo*, produced maximal (for all compared lines of different origin) sFas quantities without significant variations of expression of the $[H_2O_2^{CA} + PGE^S]$-phenotype (Table 3).

It seemed natural to address the question: how did the increase of sFas production influence the production of the membrane-bound form of this protein (mFas)? Since the method of mFas detection (cellular ELISA) applied in our study did not allow the detection of the absolute quantities of this protein in compared cell lines, in Table 4 we present data on the level of mFas production (in relative units) in parental cell lines and selectively in a set of their *in vivo* acquired variants. As can be seen from the data, the highest level of mFas production was observed in the cells of parental STHE line; it remained almost the same in STHE-bcl-2 cell line, and was considerably lower in HET-SR cells. All variant TC lines of different origin (totally 8) studied in this set of experiments were characterized by significant, 3-6-fold, reduction of mFas production after *in vivo* selection for 4-20 days.

Thus, comparison of the results of all four series of experiments indicate that several-fold increase of production of the soluble Fas form during *in vivo* selection of TC coincides with abrupt reduction of production of its membrane-bound form. Possibly, the mechanism of the increase of sFas production is connected with the switch of the alternative splicing to the soluble Fas form. It seems of interest to investigate in further studies what are the immunological consequences of this change in the balance between mFas and sFas isoforms, in particular, for the property of TC to protect themselves from CTA of NK cells and other effectors of the immune system that express FasL.

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