TRANSFORMING GROWTH FACTOR β1 PROTECTS HUMAN LUNG ADENOCARCINOMA CELLS FROM HYPERTHERMIA–INDUCED APOPTOSIS

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ЗНАЧЕНИЕ ТРАНСФОРМИРУЮЩЕГО ФАКТОРА РОСТА β1 В ЗАЩИТЕ КЛЕТОК АДЕНОКАРЦИНОМЫ ЛЕГКИХ ЧЕЛОВЕКА ОТ АПОПТОЗА, ОБУСЛОВЛЕННОГО ГИПЕРТЕРМИЕЙ

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Transforming growth factor β (TGF-β) plays a dual role in tumorigenesis, being involved in growth control in normal tissues and benign tumors, while many malignant and especially metastatic tumors were found to be resistant to TGF-β. Action we show here that human lung adenocarcinoma A-549 cells which are growth-inhibited by TGF-β1 are also protected by this cytokine from following hyperthermia-induced death. At the same time human larynx carcinoma HEp-2 cells resistant to TGF-β1 growth inhibitory effect could not be protected by TGF-β1 from heat shock killing. The data obtained suggest that the growth inhibition of carcinoma cells by TGF-β1 could be responsible for the protective effect of this cytokine against the hyperthermia-induced cell death.
***Key Words*: transforming growth factor β, hyperthermia, apoptosis.**

Трансформирующий фактор роста β (ТФР-β) принимает участие в контроле роста клеток непораженных тканей и доброкачественных опухолей. В то же время многие злокачественные и особенно метастазирующие опухоли являются резистентными к действию ТФР-β1. Предварительная обработка ТФР-β1 защищает клетки аденокарциномы легких человека А-549 (чувствительные к ингибированию роста с помощью ТФР-β1) от апоптоза, обусловленного гипертермией. На клетках карциномы гортани человека НЕр-2, которые являются резистентными к ингибированию роста под действием ТФР-β1, подобный защитный эффект ТФР-β1 не выявлен. Полученные данные позволяют предположить, что ингибируя рост опухолевых клеток, ТФР-β1 может защищать клетки от цитотоксического действия гипертермии.
***Ключевые слова*: трансформирующий фактор роста β, гипертермия, апоптоз.**

Transforming growth factor β (TGF–β) is a widely spread multifunctional cytokine. A big superfamily of TGF–β–related proteins exists and TGF–β1 is the most prevalent and well studied [1–4]. Being involved in the regulation of different cellular functions, TGF–β1 could be found in practically all studied mammalian cells [5, 6]. Inhibition of cell proliferation (in particular, cells of epithelial origin) is one of the most prominent activities of TGF–β1 [3]. The role of TGF–β as a suppressor of the growth and activity of immune cells is also well documented [4, 7]. As most tumor cells are known to produce increased amounts of TGF–β1 its role in tumor–induced immunosupression was also suggested [7].

TGF–β plays a dual role in oncogenesis. At the early stages TGF–β could serve as a negative modulator of cellular growth, while at the late stages TGF–β over–expression could aggravate the malignant phenotype [8, 9]. Such a dual role of TGF–β could be explained in part by the loss of responsiveness to growth inhibitory signals [1, 2] due to functional inactivation or loss of expression of TGF–β receptors [1, 2] and/or the components of the intracellular signalling Smad proteins by deletion or point mutations of the appropriate structural genes [10, 11], or through negative regulation by the oncogenic Ras protein [12].

Local hyperthermia was shown to be effective in tumor treatment [13], although in some cases tumors are resistant to heating. It was proposed that the elevated expression of heat shock proteins found in many tumors [14] or the peculiarities of tumor perfusion rate and energy production [15] can be of importance here and explain resistance of tumors to hyperthermia.

Here we describe a novel mechanism of the resis–tance of tumor cells to hyperthermia consisting in the alterations in cell sensitivity to TGF–β.

MATERIALS AND METHODS

Cells. Human lung adenocarcinoma A–549 cells were obtained from Research Institute of Carcino–genesis, Oncological Scientific Center of the Academy of Medical Sciences of Russian Federation (Moscow, Russia). Human larynx carcinoma HEp–2 cells were obtained from R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National

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Academy of Sciences of Ukraine (Kyiv, Ukraine). The cells were cultured in Dulbecco modified Eagle's medium (DMEM, Flow Labs, Scotland) supplemented with 10% fetal calf serum (FCS, Dialab, Lviv, Ukraine). Proportion of dead cells was determined after their staining with 0.01% (w/v) Trypan blue solution.

Heat treatment. Culture flasks with subconfluent cell layers were heated in a water bath at different temperatures for 30 min. At specified times after the termination of hyperthermia cells were collected and seeded in semisolid agar–supplemented medium as indicated below. In some experiments the cells were treated additionally with TGF–β1.

Analysis of the anchorage–independent growth. 40 mm plastic Petri dishes (Lenmedpolymer, Saint–Petersburg, Russia) were covered with 1 ml DMEM, supplemented with 10% FCS and 0.5% agar (Difco, USA). After hardening of the agar support 1 ml of cellular suspension (10^4 cells) in full culture medium supplemented with 0.33% agar was added to each dish. Cells were further incubated in the presence of TGF–β1 (10 ng/ml, R&D Systems, Indianapolis, USA) or without TGF–β1 for 14 days in 5% CO_2/95% air atmosphere at 37°C and 100% humidity. Cell colonies not less than 50 mm in diameter were counted.

DNA isolation and electrophoresis. DNA was isolated and analysed as described by Herrmann *et al*. [16]. $5 \cdot 10^6$ cells were harvested, pelleted, and resuspended in 50 μl of 20 mM EDTA, 50 mM Tris–HCl (pH 7.5) containing 1% NP–40 (lysis buffer). Samples were centrifuged for 5 min at 1600 g, and pellets were resuspended in lysis buffer. SDS (Serva, USA) (final concentration 1%) and RNase A (Sigma, USA) final concentration 1 mg/ml) were added to each sample which were then incubated for 2 h at 37°C. After that, proteinase K (Sigma, final concentration 1 mg/ml) was added to each sample which was then incubated for 2 h at 37°C. 10 M ammonium acetate (1/2 of sample volume) was added to each sample and DNA was precipitated with 2 volumes of isopropanol at –20°C overnight. Samples were centrifuged for 15 min at 10 000 g, pellets were air dried, dissolved in TE–buffer (10 μl) and loaded into the wells of 1% (w/v) agarose gel. Electrophoresis was carried out in 0.001 M EDTA, 0.04 M Tris–acetate buffer (pH 8.0) until marker dye migrated 6–7 cm. Electrophoregrams were examined under UV light and photographed.

RESULTS AND DISCUSSION

Two different human epithelial tumor cell lines were used in this study: lung adenocarcinoma A–549 cells known to be susceptible to TGF–β growth inhibition [17, 18] and larynx carcinoma HEp–2 cells resistant to TGF–β inhibitory effect [18]. As shown in Fig. 1, the anchorage–independent growth of A–549 cells is inhibited 3.5 times by TGF–β1 (10 ng/ml), while HEp–2 cell colony forming efficiency was not affected by this cytokine.

We studied the effects of different hyperthermia regimes on the viability of HEp–2 cells. 30 min treatment at 47°C was shown to be a minimal exposure causing

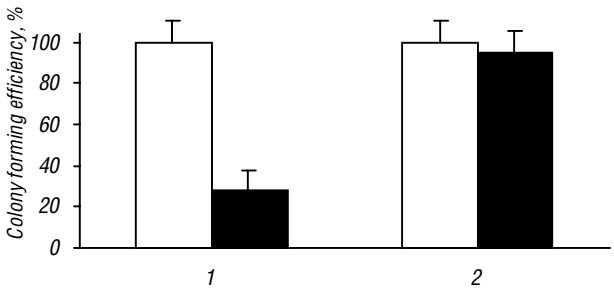

Fig. 1. Effect of TGF–β1 (10 ng/ml) on colony forming efficiency in semisolid agar: *1* — human lung adenocarcinoma A–549 cells; *2* — human larynx carcinoma HEp–2 cells. Cells were grown with (■) or without (□) TGF–β1

death of about 40% of cells in 24h after the termination of heating. Milder hyperthermia (45°C, 30 min) induced 40% cell death only in 72 h (data not shown). A–549 cells were found to be much more sensitive to the hyperthermia with 40% cell death attaining after 30 min treatment at 45°C (Fig. 2).

Pre–treatment of A–549 cells with TGF–β1 (5 ng/ml) for 24 h protected these cells from the following heating (45°C, 30 min) (Fig. 2, *a*). Nevertheless, continuous presence of TGF–β1 in culture medium after heat treatment masked such protective effect (Fig. 2, *a*).

In the same set of experiments TGF–β1 did not change the effects of hyperthermia on HEp–2 cells (Fig. 2, *b*). These data are in accordance with the resistance of HEp–2 cells to the growth–inhibitory effects of TGF–β1 (Fig. 1).

The mechanisms responsible for the loss of responsiveness of HEp–2 cells to TGF–β1 anti–proliferative effects are now under study. It is possible that functional inactivation or loss of expression of TGF–β1 receptors and/or Smad proteins which are known as the components of intracellular signalling pathway for the members of TGF–β superfamily are involved in the development of resistance to TGF–β1 [1–3]. Such inactivation of TGF–β signalling pathway components has been already shown in different tumors of epithelial origin [10, 11, 19].

While incubation with TGF–β1 has not resulted in any significant changes in the proportion of Trypan blue–stained cells (Fig. 2), we detected TGF–β1–induced apoptotic DNA fragmentation in A–549 cells incubated 72 h in the presence of TGF–β1 (Fig. 3). Similar fragmentation of DNA was induced by heat shock (44°C, 30 min) following 48 h, and especially 72 h incubation. In a full accordance with data presented in Fig. 2, pretreatment of these carcinoma cells with TGF–β1 (10 ng/ml) protected them from killing effect of hyperthermia (Fig. 3) as was evident by lower intensity of DNA fragmentation. It should be noted that treatment of A–549 cells with TGF–β1 immediately after heat shock significantly enhanced DNA fragmentation. This effect can explain why we have not observed the protective action of TGF–β pre–treatment when TGF–β1 was present in culture medium after heat shock.

Distinct DNA ladder shown for A–549 cells (Fig. 3) suggests that both TGF–β1 and heat shock (44°C,

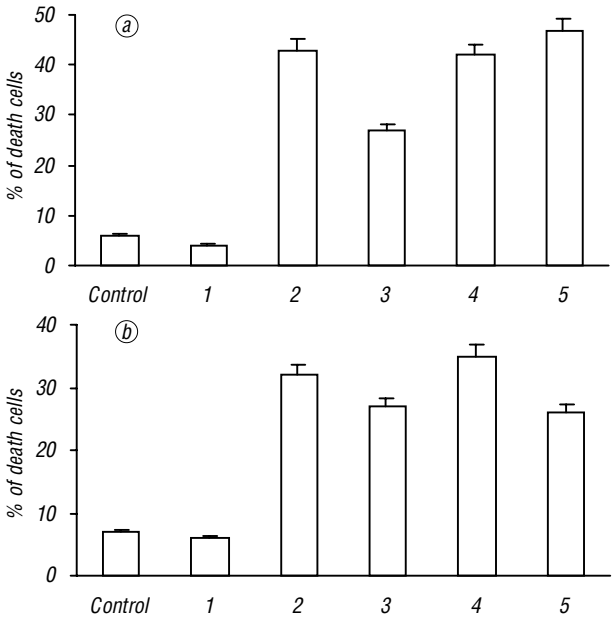

Fig. 2. Effect of hyperthermia, TGF–β1 and their combination on the viability of A–549 (*a*) and HEp–2 (*b*) cells grown in the monolayer culture:
1 — TGF–β1 (5 ng/ml); *2* — hyperthermia (45°C, 30 min for A–549 cells and 47°C, 30 min for HEp–2 cells); *3* — cells were grown in the presence of TGF–β1 (5 ng/ml), subjected to heat shock and incubated for 36 h in the presence of TGF–β1 (5 ng/ml); *4* — cells were grown in the presence of TGF–β1 (5 ng/ml), subjected to heat shock and incubated for 36 h without TGF–β1; *5* — cells were grown without TGF–β1, subjected to heat shock and incubated for 36 h in the presence of TGF–β1 (5 ng/ml)

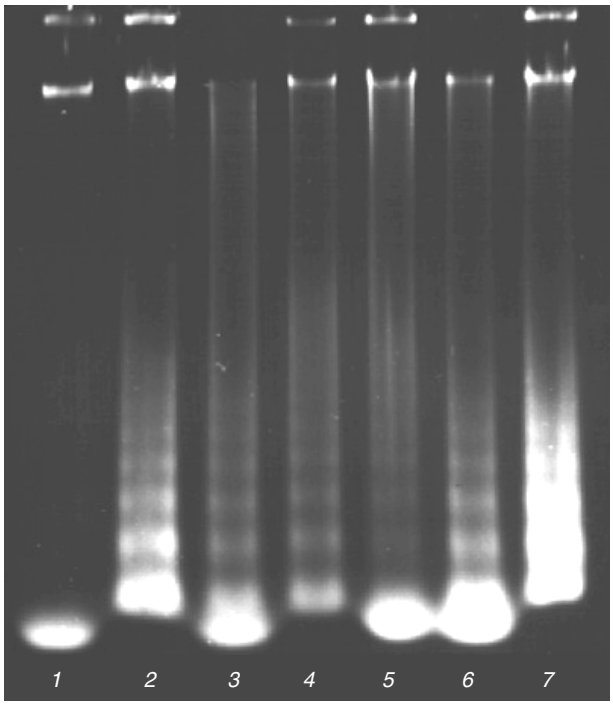

Fig. 3. DNA fragmentation in human A–549 cells upon different condition of culture: *1* — untreated cells; *2* — cells were incubated with TGF–β1 (5 ng/ml) for 72 h; *3* — cells were subjected to heat shock (44°C, 30 min) and further incubated for 48 h without TGF–β1; *4* — cells were subjected to heat shock (44°C, 30 min) and further incubated for 72 h without TGF–β1; *5* — cells were pretreated with TGF–β1 for 24 h, subjected to heat shock (44°C, 30 min) and further incubated for 48 h without TGF–β1; *6* — cells were subjected to heat shock (44°C, 30 min) and further incubated for 48 h in the presence of TGF–β1; *7* — cells were subjected to heat shock (44°C, 30 min) and further incubated for 72 h in the presence of TGF–β1

30 min) induced cell death preferentially in apoptotic way. More severe heating of A–549 cells resulted in increasing appearance of smear indicative of randomly destroyed DNA overlapping characteristic ladder DNA fragmentation (data not shown). Such pattern of resolution for DNA degradation products from A–549 cells heated at 45°C for 30 min suggests that the indicated treatment induced necrotic death of large proportion of cells in addition to cells dying in apoptotic way. The severity of this treatment can also explain why we were not able to observe an enhancement of cell killing after heat shock (45°C, 30 min) in the case when cells were further incubated in the presence of TGF–β1 (Fig. 2).

TGF–β1 was shown to block cell cycle at the boundary between late G1 and S–phases by dephosphorylation of retinoblastoma protein [3]. It was also found that the resting cells are much less sensitive to different agents including heat shock than the cells in S–phase or mitotic cells [20]. Thus, one can speculate that such growth–arrested by TGF–β1 cells are in more stable state than actively growing cells in spite of the fact that TGF–β by itself can induce the apoptotic cell death (Fig. 3, [2, 3]). Taking into account high level of TGF–β1 production by numerous tumor cells [7], our results suggest that the sensitivity of each particular tumor cells (or cell population within tumor) to TGF–β1 growth inhibitory effects can serve as an important indicator defining the individual sensitivity of these cells to killing effect of hyperthermia.

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