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FROM THE DEPARTMENT OF INTERNAL MEDICINE AND THE INSTITUTE OF PATHOLOGY, WILHELM-PIECK-UNIVERSITY, ROSTOCK, EAST GERMANY, AND THE DEPARTMENT OF CELL PATHOLOGY, INSTITUTE OF PATHOLOGY, POMERANIAN MEDICAL ACADEMY, SZCZECIN, POLAND.

DNA CYTOMETRIC AND HISTOLOGIC FINDINGS IN MOUSE TUMORS (BP AND S 180) WITH DIFFERENT RESPONSE TO TREATMENT WITH TUMOR NECROSIS FACTOR

J. RYCHLY, E. KNIPPEL, A. KRYGIER-STOJALOWSKA, H. NIZZE, M. KUCHNIO and S.-K. KRAEFT

Abstract

DNA cytometric and histopathologic investigations were performed in two tumor models (BP and S 180) which differed in their sensitivity to tumor necrosis factor (TNF). TNF induced strong necrosis in both tumors, but only the sarcoma 180 showed total regression. After TNF administration DNA cytometry revealed in the BP tumor an increase of cells in the S-phase, and in the S 180 tumor a loss of aneuploid cell populations. Histologic examination revealed a more obvious effect of TNF on tumor blood vessels in BP tumor, whereas infiltration of inflammatory cells was observed only in the S 180 tumor. We concluded that cell infiltration may be of importance for tumor regression and that aneuploid cell populations are more sensitive to TNF treatment than eudiploid cells.

Key words: Tumor necrosis factor; mouse tumors, experimental treatment, DNA cytometry, histology.

Tumor necrosis factor (TNF-α) induces hemorrhagic necrosis in several mouse tumors and displays a cytotoxic effect on different tumor cell lines in vitro (1–3). This factor is now well characterized as a cytokine with known molecular weight, amino acid and DNA sequence which is released by stimulated monocytes and macrophages (3). Since recombinant TNF became available much progress has been made concerning its biological functions (4). Receptors on tumor cells seem to be a prerequisite for the in vitro effect but are not sufficient for cell killing (5). Recently it has been shown that TNF exerts direct antitumoral activity via selective regulation of gene expression (6). In vivo intratumoral as well as systemic administration causes necrosis and sometimes complete regression of several mouse tumors (7, 8). Whether direct cytotoxic or indirect mechanisms are involved is not clear. Several indications support the hypothesis that the in vivo effect is mediated through the host system (7, 9). We investigated the effect of TNF on two mouse tumor models which differed in their sensitivity to TNF, namely BP-tumor and sarcoma 180. We used histological investigations and DNA cytometry in order to identify some characteristics of the tumor cells which may be of importance for the sensitivity to TNF in vivo.

Material and Methods

Animals and tumors. Two mouse tumor models were used: 1) BP Ros/01 (BP) transplanted on 6-week-old female CBA mice. This sarcoma was originally induced by benzpyrene and subsequently maintained by passages of subcutaneously (s.c.) grown tumor cells. In the experiment a suspension of 2×10^5 tumor cells was injected subcutaneously into the right flank of the animals. 2) Sarcoma 180 (S 180) transplanted on 6-week-old female ICR mice. This tumor was kindly provided by Dr Shimizu, Tokyo and maintained intraperitoneally. In the experiment 1×10^6 tumor cells were injected s.c. into the right flank.

Tumor necrosis factor. We used recombinant human tumor necrosis factor (rH-TNF-α) expressed in Escherichia coli and kindly provided by Asahi Chemical Industry Co., Ltd. Tokyo. The activity in U/ml was determined on the basis of its cytotoxicity against L 929 cells in vitro. Further physico-chemical characteristics have been described earlier (10). Dilution of TNF for administration to

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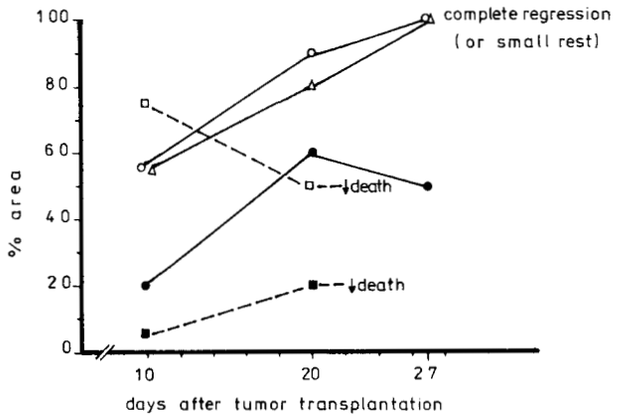

Fig. 1. Extent of necrosis estimated from the histological examination. (——): sarcoma 180; ●=untreated tumor, ○=high dose of TNF, △=lower dose of TNF, (---): BP tumor; ■=untreated tumor, □=TNF treated tumor (each point represents the mean of 3 animals).

animals was always performed with endotoxin-free diluent (Asahi Chemical Industry).

Administration of TNF. In the BP-tumor model animals (in all 9 mice) received 4000 U TNF/animal on days 7, 12, 14, 17 and 19 (in all 20000 U) after tumor transplantation by intratumoral administration. In the S 180 tumor model we used two schedules of TNF application. One group of mice (12 animals in all) received 5000 U TNF on days 1, 2, 3, 7, 8, 9, 13 and 14 (totally 40000 U) by intratumoral administration. A second group of S 180 tumor mice (12 mice in all) received 5000 U TNF/animal on days 1, 9, and 14 (in all 15000 U).

Histologic examination and cytometric DNA measurements. On days 10 and 20, and in the S 180 tumor model also on day 27, 3 animals from each group were killed and the tumors with a small margin of surrounding host tissue removed for examination. The tumor was cut in the middle and imprints for DNA measurements made. Sections of tumor tissue were fixed in 4% formalin, embedded in paraffin and stained with hematoxylin and eosin for histologic examination. This examination included estimation of the area of necrosis, morphological changes in tumor cells, changes in tumor vessels and extent of inflammatory infiltrations.

The imprints were fixed in formalin vapors and methanol and stained by Feulgen method using pararosaniline (Merk, Darmstadt). The quantitative analysis was carried out by a computer microscopic image analyzer (VEB Carl Zeiss Jena) with measurement of DNA amount, size of nucleus, mean extinction value, highest extinction value, and mean extinction value of nuclear circumference. From these measurements we also derived the chromatin compactness degree and the ratio of the nuclear circumference extinction to the nuclear center extinction. The latter parameter permitted analysis of the nucleoli, which the Feulgen method fails to stain. The diploid DNA value

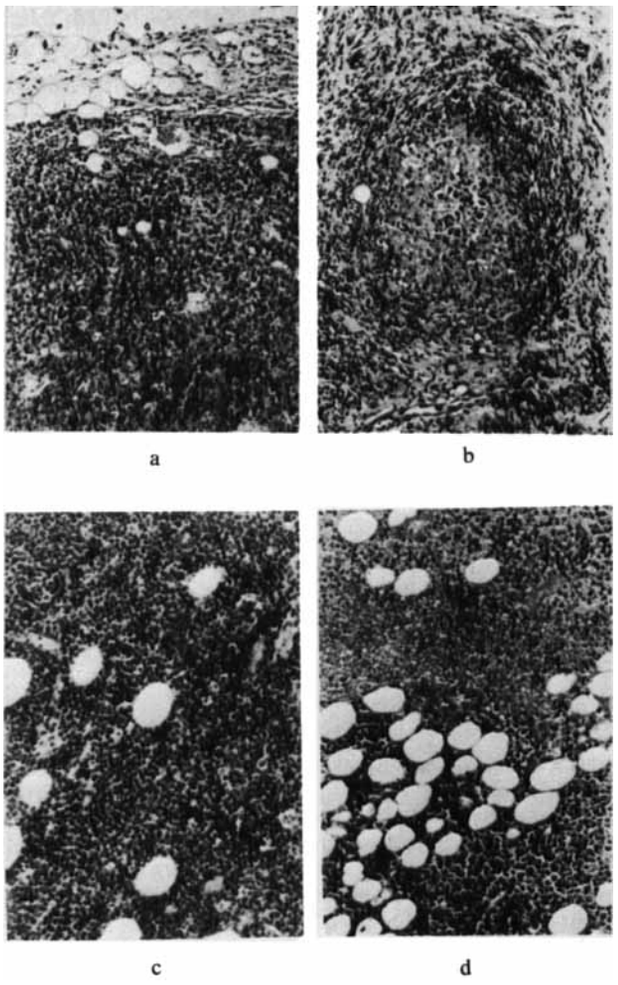

Fig. 2. a) Untreated sarcoma 180 (27 days after transplantation) without signs of regression, b) TNF treated sarcoma 180 (27 days after transplantation): single pleomorphic remnant tumor cells surrounded by lymphocytic infiltrate and fibrosed granulation tissue, c) untreated BP tumor (10 days after transplantation) without necrosis, and d) TNF treated BP tumor (10 days after transplantation). Single fibrin thrombi are seen in capillaries of necrotic tumor tissue, H.E.×170.

was estimated by measurement of normal mouse spleen lymphocytes. For estimation of the percentage of cells in G_0 phase the following parameters were taken into consideration: the diploid DNA value, the compactness degree of chromatin, and the ratio of nuclear circumference extinction to nuclear center extinction. Lower chromatin compactness degrees and presence of nucleoli (in cytometric measurements of cells stained by Feulgen method —lower value of ratio of nuclear circumference extinction to nuclear center extinction) indicate cells in the G_1 phase.

Results

The BP tumor was characterized by round tumor cells of intermediate size showing invasive growth into the

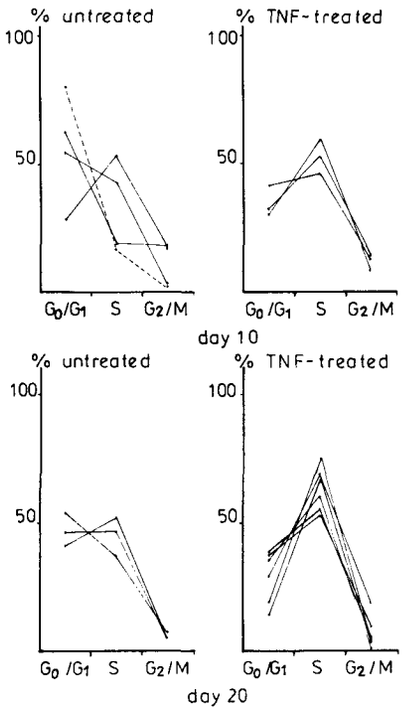

Fig. 3. Percentages of cells in the cell cycle phases G_0+G_1, S and G_2+M in the BP tumor of untreated and TNF treated tumor mice at day 10 and 20 after tumor transplantation (significant differences ($p<0.01$) of S phase cells between untreated and TNF treated animals at day 20).

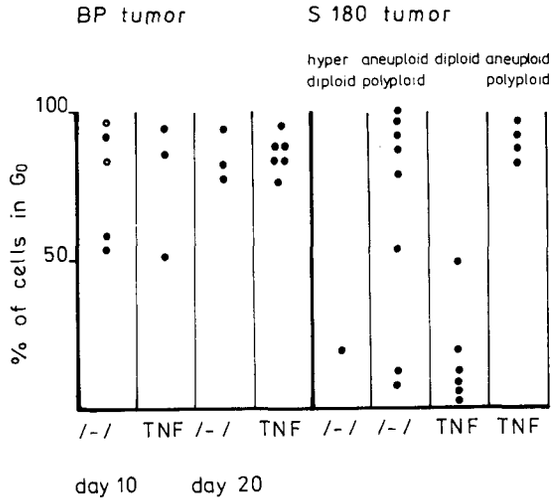

Fig. 4. Percentages of BP and S 180 tumor cells in G_0 inside the G_0+G_1 peak in untreated (–) and TNF treated (TNF) tumor mice (○=normal spleen cells).

surrounding tissues. The sarcoma 180 consisted of smaller, anaplastic tumor cells. In untreated animals spontaneous necrosis was more pronounced in S 180 tumors than in the BP tumors, where necrosis was negligible on day 10 after tumor transplantation (Fig. 1). TNF induced pronounced central necrosis in both BP and S 180 tumors.

Table

Percentages of tumors in different ploidy levels

Groups of animals	Diploid	Hyperdiploid	Aneuploid-polyploid
Untreated			
S 180 mice (n=9)	0	10	90
TNF treated			
S 180 mice (n=10)	60	0	40
Untreated			
BP mice (n=6)	100	0	0
TNF treated			
BP mice (n=9)	100	0	0

However, the tumors differed considerably regarding size reduction and regression. Despite strong necrosis the BP tumors showed no distinct size reduction after TNF and the survival time of the animals was about 22 days (similar to untreated tumor mice) (see ref. 11). In contrast, TNF treated S 180 tumors declined in size from day 20 after tumor transplantation (see ref. 12) and on day 27 the tumors had completely regressed (Fig. 2). This effect was found after both high and low doses of TNF. In both tumors TNF treatment promoted the different stages of nuclear degeneration as pyknosis and karyorrhexis. In the BP tumor the effects were mainly evident at the beginning of the experiment.

Concerning the influence on tumor vessels a moderate hyperemia was found in both tumors, but only in BP tumors could some fibrin thrombi be observed after TNF treatment. In untreated S 180 tumors some thrombi were found in the neighborhood of spontaneous necrosis during the late stage of tumor development. The tumors showed differences regarding inflammatory infiltration. In the BP tumor, no interstitial tissue was visible which could contain a cell infiltrate. In contrast, the S 180 tumor was characterized by a moderate inflammatory cell infiltrate in the stroma. On day 10 after tumor transplantation the number of infiltrating granulocytes obviously increased after TNF administration. At the end of the experiment, fibroblasts appeared at the rim of the organized tumors (Fig. 2).

DNA cytometry revealed striking differences of ploidy pattern between the two tumor types. The BP tumor cells were diploid, whereas the sarcoma 180 contained also aneuploid cell populations and, with one exception, the cells had at least two DNA stem lines. The S 180 tumor could thus be defined as aneuploid and aneuploid-polyploid (Fig. 3 and Table). TNF treatment changed the DNA histograms in both tumor models. In the TNF treated BP tumors we observed a significantly increased percentage of cells in the S-phase on day 20 after tumor transplantation (Fig. 3). Concerning the G_0+G_1 peak, the proportion of G_0 cells varied between different animals but no clear influence of TNF therapy was discernible (Fig. 4). In the

S 180 tumors a decrease of the aneupolyploid cell populations was observed after TNF administration (Table). While in untreated animals 90% of the tumors were aneupolyploid, only 40% of the tumors had this DNA stem line type after TNF treatment. The percentage of G_0 cells in the first DNA stem line of the DNA histogram is shown in Fig. 4. In untreated tumors the percentage of G_0 cells varied considerably. In TNF treated tumors with an exclusively diploid cell population nearly all cells of the G_0+G_1 peak were in the G_1-phase, whereas in the tumors with a DNA-aneupolyploid cell type, the cells in G_0 prevailed.

Discussion

TNF induced strong necrosis in two tumor models, but only in one of them complete regression occurred. Although the mechanisms of the antitumor action of TNF in vivo are unknown, several findings argue for indirect mechanisms (7, 9). Our histological examination confirmed other reports that TNF may affect the tumor blood vessels, leading to circulatory stasis and hemorrhagic infarction of the tumor (7, 13, 14). This finding agrees with the observation that TNF affects hemostatic properties of endothelial cell cultures in vitro (15, 16). The presence of thrombi in the TNF-treated BP tumor indicated a stronger effect on vessels of this tumor than in the S 180 tumor. As the BP tumor showed no regression in contrast to the S 180 tumor, the TNF effect on the vascular system may be important with regard to necrosis but does not seem to be decisive for the regression of the tumor.

In the S 180 tumor, infiltration of inflammatory cells was observed in the tumor stroma. After TNF administration, granulocytes increased in number at the beginning of the experiment, whereas in the BP tumor no cell infiltration was detected. Therefore, it may be suggested that inflammatory cells play a role in the regression of the tumor. Asher et al. (7) found that only tumors infiltrated with inflammatory cells, mainly granulocytes, regressed, although granulocyte infiltration was not augmented after TNF treatment. Recently, the effect of TNF on granulocytes in vitro has been extensively studied (17–19); obviously TNF activates and induces several granulocyte functions, e.g. the adherence to epithelial cells (17). These functions may be related to an antitumor activity. From earlier experiments it was concluded that the immune system is most probably involved in the tumor regression induced by TNF (20), but no direct evidence of immunological mechanisms exists so far.

Sensitivity of tumors to therapeutic agents depends on different properties of the tumor cells. Ploidy level and the proportion of cycling cells may influence the sensitivity to anticancer therapy (21–23). Reports on the influence of TNF on the cell cycle in vitro are ambiguous. Darzynkiewicz et al. (24) found an increase of cells in the G_2+M phase, whereas a block of cells in G_1 phase was postulated by Nobuhara et al. (8). We observed that in the BP tumor TNF treatment caused an accumulation of cells in the S-phase. This difference compared with the finding in vitro probably gives a further hint for an indirect action of TNF in vivo. Regardless of the reason for an increased number of cells in the S-phase, this observation suggests the use of TNF in combination with anticancer drugs, which are specifically effective in the S-phase.

In the S 180 tumor, which was heterogenous with respect to the ploidy level, the DNA histograms suggested a higher sensitivity of the aneuploid tumor cells to TNF. This might also be supported by the fact that the BP tumor, which showed no regression, contained only diploid cells. In human malignancies the association between ploidy pattern and sensitivity to treatment seems to vary between different types of neoplasms (21, 25). In addition to the ploidy pattern the proportions of resting and cycling cells and the proliferation rate may be of importance for the response to TNF treatment. The remaining diploid cells in the S 180 tumor after TNF treatment included a very low proportion of G_0 cells which may indicate that nearly all cells proliferated. The BP tumor cells have a 4 times higher ^{3}H-thymidine incorporation rate than the S 180 tumor cells (own unpublished results). Therefore, one may argue that, with a high proportion of cycling cells and high proliferation rate, the antitumor mechanisms induced by TNF might not be sufficient for regression of the tumor.

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