Human soluble tumor-associated antigens promote the suppression of rat mammary tumors by 5-fluorouracil and stimulate the functional activity of immune organs: Experimental and morphological studies

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Abstract. This study examined whether the soluble 66 and 51 kDa tumor-associated antigens (sTAA) could promote suppression by the anticancer drug 5-fluorouracil (5-Fu) of chemically induced mammary tumorigenesis, and which, if any, morphological changes in the immune organs accompany this treatment. Dimethylbenzanthracene (DMBA, 8 mg/rat, twice) was used to induce mammary tumors. After the appearance of many large tumors, the preparations of sTAA and 5-Fu, alone or in combination, were administered in weekly doses, for 4 weeks. The following groups of mammary tumor-bearing rats were studied: 1) control non treated rats, 2) rats treated with sTAA, 3) rats treated with 5-Fu, 4) rats treated with 5-Fu and sTAA. The experiment was terminated when tumors in 70% of control rats became ulcerous. Treatment with sTAA alone significantly decreased tumor yield and their total area relative to controls. Both of these parameters showed an even larger significant decrease after treatment with 5-Fu, and the most marked decrease was obtained after the combined treatment with 5-Fu and sTAA. The experiment was terminated when tumors in 70% of control rats became ulcerous. Treatment with sTAA alone significantly decreased tumor yield and their total area relative to controls. Both of these parameters showed an even larger significant decrease after treatment with 5-Fu, and the most marked decrease was obtained after the combined treatment with 5-Fu and sTAA. Results demonstrated that not only do sTAA have tumour-suppressive properties, they also enhance the anticancer effects of 5-Fu and prevent its toxic side effects. Morphologically, the treatment with sTAA was manifested in a significant increase in the size of the spleen follicles and mantle layer compared to control rats with large tumors. The treatment with 5-Fu decreased the sizes of almost all areas of the spleen compared to control rats, whereas the combined treatment with 5-Fu and sTAA increased all these parameters to the levels found in rats treated with sTAA alone. The total areas of the cortex and paracortex in the lymph nodes increased after treatment with sTAA. Treatment with 5-Fu alone resulted in a significant decrease of these areas which, as seen in the spleen, increased after combined treatment with 5-Fu and sTAA. Similar changes were seen in the areas of the separate lymph node zones. We concluded that the addition of sTAA to conventional tumor chemotherapy regimens has a remarkable synergistic effect on mammary tumors leading to curative antitumor responses of the host's immune organs.

Introduction

Cancer chemotherapy is well known to have disastrous toxic side effects (1,2), and many studies have been performed to eliminate, or at least decrease such secondary effects. In this respect, cancer vaccines derived from tumor cells and stimulating a wide spectrum of immune responses against a variety of tumor antigens have proven useful. Tumor-associated antigens (TAA), presenting as a part of the major histocompatibility class I complex on either the tumor cell itself or on antigen-presenting cells, are capable of inducing tumor-specific cytotoxic T lymphocytes (3). We have previously found that vaccinating rats with soluble 66 and 51 kDa tumor-associated antigens (sTAA) inhibits mammary gland tumorigenesis, promotes the tumor-suppressor effect of anticancer drugs such as cyclophosphamide (CPA) and tamoxifen, and prevents their toxic side effects (4,5). We have also shown that CPA decreases the size and cell content of splenic follicles, related to the production of B cells, and to a lesser extent of the perialterial lymph sheath, related to the production of T cells (6). Additional therapy with sTAA restored activity in the splenic zones producing these cells. A similar effect of CPA and sTAA was found in lymph nodes with the accumulation
of B lymphocytes in the primary and secondary follicles, and of T lymphocytes in the paracortical zone (6). We suggest that inhibition of the functional activity of the immune system is one of the main reasons for the toxic effects of chemotherapeutic drugs, such as CPA and tamoxifen, and that the tumor-suppressive antitoxic effect of sTAA results from their activation of B- and T-lymphocyte production in this system, particularly in the spleen and lymph nodes. In the present study, we analyzed whether sTAA have similar effects on an other anticancer drug, 5-fluorouracil (5-Fu), widely used in oncological practice for the treatment and prevention of breast cancer (7,8) despite its high toxic side effects (1,2,9).

Materials and methods

Animals. All experiments were carried out in accordance with guidelines established by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem. Sixty 8-week-old Sprague Dawley female rats (Harlan Labs., Rehovot, Israel) were housed in polypropylene cages under a 14 h/10 h light/dark regimen at 22±2°C, and were provided standard lab chow and tap water ad libitum.

Tumorigenic experiments. Tumors were induced with 9,10-dimethyl-1,2-benz(o)anthracene (DMBA; Sigma, St. Louis, MO). Each animal received two intragastric feedings of DMBA, a week apart, at a dose of 8 mg dissolved in 0.5 ml corn oil; thus, the total amount of carcinogen was 16 mg/rat.

Antigen preparation. Some of the rats were treated with a preparation of sTAA containing mainly (over 90%) two low-molecular-mass proteins of 66 and 51 kDa in an approximate 1:3 ratio. These sTAA were isolated from the serum of patients with breast tumors using commercial columns for affinity chromatography (BioRad P-60, BioRad Co, Melville, NY) (10). Briefly, sTAA were separated in a column equilibrated with 0.01 M Tris buffer using 0.15 M NaCl, pH 7.5. sTAA were eluted in 0.1 M glycine buffer, pH 2.5, and the pH of the eluted protein was immediately adjusted to 7.5. The partially purified mixture of sTAA showed two bands at 66 and 51 kDa, representing proteins whose molecular masses were determined by SDS-PAGE under non-reducing conditions and by mass spectrometry.

Treatments of tumor-bearing rats. After the appearance of tumors, their growth to 15-20 mm in diameter and a yield of 2.6-3.4, 9-10 weeks after the second injection of DMBA, the tumor-bearing animals were divided into five groups. Rats in control groups 1 and 2 (n=20, small and large tumors, respectively) received no additional treatment and served as the untreated control groups. Rats in group 3 (n=12) were fed weekly with the commercial anticancer drug 5-Fu (Pharmachemie BV, Haarlem, Holland). The drug (15 mg/rat) was dissolved in 0.5 ml distilled water and administered weekly for 4 weeks (11). Rats in group 4 (n=12) were injected subcutaneously with a preparation containing 50 μl sTAA dissolved in 0.5 ml phosphate-buffered saline, weekly for 4 weeks. Rats in group 5 (n=12) were treated with a combination of 5-Fu and sTAA. All dead rats showed distinct signs of hypotrophy to cachexia as a result of advanced
tumorigenesis. Cases involving intercurrent causes of death were not included in this work.

Scoring and analysis. During the course of the experiment, all animals were scored for mammary tumors, which were palpated, counted, and measured. The experiment was terminated 14 weeks after the first introduction of DMBA, when tumors in 70% of control rats became ulcerous. All surviving rats were sacrificed by intraperitoneal injection of pentobarbital (0.2-0.3 ml/rat); the tumors were measured and excised for routine pathological examination. The following tumorigenic indicators were evaluated in each rat: the number and dimensions of the tumors, the number of malignant tumors, and the yield coefficient, namely, the ratio of total number of tumors to the number of tumor-bearing rats. Tumors were considered malignant if they were firmly attached as a result of having infiltrated the surrounding tissue, often with dermal involvement or ulceration.

Morphological study. The spleen and submandibular or axillary lymph nodes were taken from seven to eight rats from each group for morphometric analysis. All histopathological studies were performed using standard procedures, with uniform conditions of fixation and staining of 3-μm sections with hematoxylin and eosin (H&E). The areas of different zones in the spleen (transverse sections) and lymph nodes (central sections) were counted per 10,000 μm² with an ocular grid at a magnification of x150. Our previous studies have shown that this parameter reflects the number of lymph cells in the lymph organs studied (6). In the spleen, the following zones were measured: white pulp with follicles, mantle layer and germinal centers (containing mainly B cells), periarterial lymph sheaths (PALS, containing mainly T cells), marginal zone, and red pulp. In lymph nodes, the following zones were measured: cortex with primary follicles, similar to the mantle layer of the spleen, and secondary follicles, similar to the germinal centers of the spleen (both containing mainly B cells), paracortex (containing mainly T cells), and medulla.

Statistical analysis was performed using one-way ANOVA. Pairs of means were compared using the Tukey HSD test.
Results

Tumorigenic experiments. High doses of carcinogen caused a very intensive rate of tumorigenesis in rats (Figs. 1 and 2). In control rats of group 2, this was manifested in a high rate of tumor growth, their huge size, and a high percentage of malignant tumors. The treatment with sTAA alone significantly decreased, compared to controls, the yield of tumors (Fig. 1) and also the total area of tumors, especially when it was evaluated per rat (Fig. 3). The parameters studied both showed further significant decrease after the treatment with 5-Fu and especially after the combined treatment with sTAA and 5-Fu.

Morphological analysis

Spleen. The treatment with sTAA showed a significant increase (up to 121%) in the size of the follicles and mantle layer compared to untreated control rats with large tumors (Table I). The treatment with 5-Fu alone inhibited growth of these areas, except the medulla, and the combined treatment with 5-Fu and sTAA partially restored their size.

Lymph nodes. The total areas of cortex and paracortex in the lymph nodes increased, after treatment with sTAA, up to 113% and 123% of controls (Table II). Treatment with 5-Fu alone resulted in a significant (up to 63%) decrease in these areas, which increased after the combined treatment with 5-Fu and sTAA (up to 78% of control group 2). Similar changes were seen in the areas of separate lymph node zones: the treatment with 5-Fu alone inhibited growth of these areas, except the medulla, and the combined treatment with 5-Fu and sTAA partially restored their size.

Discussion

The discovery and characterization of several TAA found in various tumors have facilitated more accessible therapeutic
These TAA represent cellular products (mostly proteins) that are preferentially expressed by tumor cells, although in many cases they are also found to some extent (but usually in lower amounts) in normal tissue (12,13).

Results of our previous observations, as well as those of the present study, show that in poorly immunogenic and aggressive systemic tumor models, simple therapy with either sTAA or anticancer drugs has variable efficacy, but neither therapy alone is able to cure the high percentage of tumor-bearing animals (14). However, when both systemic immunotherapy and chemotherapy are combined, mammary tumors are rejected and treated animals are cured at a high percentage (4,5). Similar results have been reported in the literature regarding immunotherapy of murine cancers with soluble B7-immunoglobulin G (13,15).

We have previously found that vaccination of rats with the sTAA suppresses mammary gland tumorigenesis and promotes the tumor-suppressor effect of the cytotoxic anticancer drug CPA (4). These data are in accordance with observations of others showing that CPA in combination with tumor necrosis factor-α induces a 60% long-term survival rate among lymphoma-bearing mice (15). We also showed that vaccination could enhance the tumor-suppressive activity and prevent toxic side effects of the hormone-related anticancer drug tamoxifen (5).

In the present study, we found that sTAA have similar effects also on 5-Fu. The treatment with sTAA alone significantly decreased, compared to controls, the yield of tumors and their total area. Both parameters showed a further significant decrease after the treatment with 5-Fu and especially after the combined treatment with sTAA and 5-Fu.

Morphologically, the treatment with 5-Fu inhibited the sizes of spleen and lymph-node structures connected with B-cell production - a manifestation of the toxic effect of chemotherapy. The combined treatment with 5-Fu and sTAA partially neutralized the toxic effect of 5-Fu on the immune organs and increased all of these parameters (white pulp in the spleen, cortex and paracortex in the lymph nodes) to their corresponding levels in rats treated with sTAA alone.

We have previously found that treatment with CPA decreases the size and cell content of splenic areas related to the production of B and T lymphocytes, and inhibits the production of CD4+ and CD8+ T cells in the spleen (6). Addition of sTAA restores activity in the splenic zones producing these cells. Similar effect of combining CPA and sTAA was found in the lymph nodes with respect to the accumulation of CD8+ and CD4+ lymphocytes in the paracortical zone (6). This is in accordance with observations of others showing the paracortex as an area of T-cell production (16). The involvement of lymph nodes in colorectal carcinoma has been caused to procure natural T cells to respond against TAA (17). We concluded that the addition of sTAA to conventional tumor chemotherapy regimes has a remarkable synergistic effect on mammary tumors, leading to curative T-cell-dependent antitumor responses (6 BH, 01b).

Various mechanisms may contribute to the synergy observed in combined therapy, opposed to the limited efficacy of single therapies, as observed in our studies. Chemotherapy alone significantly reduces the carcinogenic burden, but cannot eradicate minimal residual disease. Apparently, this is the reason that chemotherapy-treated animals eventually succumb to lethal tumors, a situation resembling the relatively short duration of remission observed in human breast cancer patients with partial remission following induction chemotherapy (13,14,18). The efficacy of the combined regimen suggests that sTAA molecules probably strengthen antigen-containing cell-T cell interactions during antigen presentation, which in combination with chemotherapy-mediated tumor reduction can lead to curative immune responses by the host's immune organs.

A major potential advantage to using sTAA as an adjuvant to chemotherapy is its extremely high safety profile. The protein has not shown any in vivo toxicity, even with injections of 500 μg to rats or 200 μg to mice (Kossoy, unpublished data). Combination immunotherapy - chemotherapy is an emerging form of cancer treatment. With the addition of an immune-boosting agent that, in principle, forces 'provoked' immunity, conventional cancer therapy could conceivably be
made more effective without increasing its toxicity (18,19). This may be manifested in a greater durability of response rather than a higher absolute clinical response rate.

It is anticipated that individual immunomodulatory compounds will not be synergistic with anticancer drugs, owing to differential immunosuppressive effects. In this respect, the high safety profile of combining sTAA with chemotherapy in our experiments, together with its therapeutic effect, have directed our efforts toward the development of strategies for clinical evaluation.

References

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