RADIOCURABILITY BY TARGETING TUMOR NECROSIS FACTOR ALPHA USING A BISPECIFIC ANTIBODY IN CARCINOEMBRYONIC ANTIGEN TRANSGENIC MICE

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ABSTRACT

Purpose: Tumor Necrosis Factor α (TNFα) enhances radiotherapy (RT) killing of tumor cells in vitro and in vivo. To overcome systemic side-effects, we used a bispecific antibody (BsAb) directed against carcinoembryonic antigen (CEA) and TNFα to target this cytokine in a CEA-expressing colon carcinoma. We report here the evaluation of this strategy in immunocompetent CEA-transgenic mice.

Methods and Materials: The murine CEA-transfected colon carcinoma MC-38 was used for all experiments. In vitro, clonogenic assays were performed after RT alone, TNFα, and RT+TNFα. In vivo, mice were randomly assigned to treatment groups: control, TNFα, BsAb, BsAb+TNFα, RT, RT+TNFα, RT+BsAb+TNFα. Measurements of endogenous TNFα mRNA levels and evaluation of necrosis (histology evaluation) were assessed per treatment group.

Results: In vitro, combined RT+TNFα showed a significant decrease in the survival fraction at 2 Gy as compared with RT alone (p < 0.00001). In vivo, we observed five complete responses out of 10 treated mice (50%), 2/10 (20%), 2/11 (18.2%), and 0/12 (0%) in the RT+BsAb+TNFα, RT+TNFα, RT alone, and control groups, respectively. This difference was statistically significant when TNFα was targeted with the BsAb (p = 0.03). Addition of exogenous TNFα to RT significantly increased endogenous TNFα mRNA level particularly when TNFα was targeted with the BsAb (p < 0.01). Percentages of necrotic area were significantly augmented in the RT+BsAb+TNFα group.
**Conclusion:** These results suggest that targeting TNFα with the BsAb provokes RT curability in a CEA-expressing digestive tumor syngenic model and may be considered as a solid rationale for clinical trials.

**Keywords:** Bispecific antibody, Tumor Necrosis Factorα, radiotherapy enhancement, CEA-transgenic mice, synergism.
INTRODUCTION

TNF\(\alpha\) is an attractive therapeutic protein with a broad range of potent anticancer effects. This multipotent cytokine is produced mainly by activated macrophages with the ability to mediate cytotoxicity of several human and murine cell lines both in vitro (1) and in vivo (2) without inhibiting normal cell growth (1). Its anti-tumor activity is not well defined, but depends on a variety of effects within the tumor microenvironment leading to selective damage to tumor-associated vessels, activation of inflammatory and immune mechanisms, tumor cell necrosis, and tumor cell apoptosis. Recently, Ruegg et al. reported evidence for the involvement of endothelial cell integrin \(\alpha v\beta 3\) in the disruption of the tumor vasculature induced by the combination of TNF\(\alpha\) and IFN-\(\gamma\) (3), required for tumor expansion and formation of metastases.

We and others have suggested in vitro additive or supra-additive interaction between TNF\(\alpha\) and ionizing radiation (4–8) as well as an enhancement of the anti-tumor effect of radiation in some murine and human tumors in vivo (6–11). The oxidative damage produced by TNF\(\alpha\) (12) may enhance cellular damage produced by ionizing radiation. In addition, TNF\(\alpha\) and radiation can induce apoptosis in target cells (13, 14) even if cells are highly resistant to the induction of radiation-induced apoptosis (15). These findings led to a phase I trial of intravenous administration of TNF\(\alpha\) in combination with ionizing radiation. In this study, some tumor responses were noted (16), but no phase II trial was performed to confirm these preliminary results.

Indeed and despite its profound cytostatic and cytotoxic effects in primary tumors, systemic toxicity induced by TNF\(\alpha\) prevented its routine use in cancer-patients. On one hand, systemic injection of TNF\(\alpha\) evaluated in different clinical
trials showed disappointing results mainly because patients were found to have significantly lower maximum tolerated doses (17, 18) as compared with mice (19, 20) corresponding to an insufficient cytotoxic dose level. These limited results were probably due to the short circulatory half-life of TNFα and its severe systemic side effects. On the other hand, studies involving regional (21–23) or intratumoral (24) injection of TNFα have demonstrated its potential for cancer therapy but only at high local concentration.

A new approach to overcome these problems consisted of the use of a bispecific antibody (BsAb) in which one arm is directed against tumor-associated antigen (TAA) and the other arm against TNFα to target this cytokine into tumors (25). This strategy has been applied in our previous studies (6–8) with the BsAb directed against carcino-embryonic antigen (CEA) and TNFα in human CEA-expressing colorectal and pancreatic xenografts treated with radiotherapy (RT). In these experiments the in vitro growth inhibitory effect of TNFα was accompanied by a marked enhancement of the radioreponse of the tumor in vivo, particularly when TNFα was concentrated in the xenografts thanks to the BsAb. One of the advantages of the BsAb strategy is the potential decrease of systemic toxicity. Nevertheless, this property could not be addressed in nude mice (6–8). In this model, the immunological action is not possible due to the absence of T cells. Interestingly, in the syngenic model, TNFα may act its effect through immunological and non-immunological mechanisms.

Therefore we used here an immunocompetent model in which tumoricidal action of TNFα could be evaluated mainly by the immunological and non immunological mechanisms such as damage to the tumor vasculature. This model corresponds to the CEA-transgenic mice expressing this antigen as in
humans (26). *In vitro* and *in vivo* synergism of combined treatment of RT and targeted TNFα with the BsAb are reported in the present study.
MATERIALS AND METHODS

Cell line and culture conditions

The murine colon carcinoma MC-38 transfected with the human CEA cell line (C15.4.3 AP), kindly provided by FJ Primus, was used for all experiments. The cells were cultured in DMEM medium (Gibco Laboratories, France) supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories, France), glutamine (300 µg/ml), fungizone (0.25 µg/ml), streptomycin (100 µg/ml), penicillin G (100 units/ml), and geneticine (0.5 mg/ml). These cells were adherent and grew as monolayers at 37°C in a humidified 5% CO₂ incubator. Cultures were checked for the absence of mycoplasma every month.

Tumor necrosis factorα and bispecific antibody

The specific activity of recombinant human TNFα (Apotech, Switzerland) was 5 × 10⁷ units/mg protein as determined by cytolysis of murine L929 cells in the presence of actinomycin D. TNFα (at a concentration of 2.5 mg/ml) was stored at −80°C until use.

BsAb was constructed as previously described (25) from the anti-CEA MAb 35A7 and the MAb tnf18 directed against TNFα.

Radiation protocols

Cells were plated in 6 ml DMEM (to ensure homogeneous energy deposition within each dish) using 60 mm Petri-dishes and irradiated with a cobalt-60 (⁶⁰Co) source (γ irradiation, ELITE 100, Theratronics) in the Radiation Therapy Department. The radiation was delivered as a single dose ranging from 2 to 6 Gy in an 11 cm × 11 cm field size at a dose rate of 0.5 Gy/min. A 3 cm polystyrene block was used under the Petri dishes during each irradiation to allow
homogeneous back-scattering γ-rays. Source-half depth distance (SHD) was initially calculated to obtain a constant dose rate of 0.5 Gy/min and monthly adapted from the 60Co source radioactivity decrease. Control cells were removed from the incubator and placed for the same period of time under the 60Co source but without radiation treatment. In the combined treatment modality studies, TNFα was added twelve hours prior to RT.

For in vivo tumor treatment, the radiation was delivered to the flank of anaesthetized mice simultaneously in a 12.5 cm × 12.5 cm field size at 6 Gy/fraction at a dose rate of 0.5 Gy/min (SHD of 158 cm), twice a week, for a total dose of 30 Gy. A 6 cm thick lead block with 8 circular apertures, 3 cm in diameter, was used so that only the tumours and the underlying normal tissues were exposed to the radiation. Radiation was measured using dosimetry films (RA711P, Agfa, Belgium).

All mice were anaesthetized, regardless of treatment group, to equalize the effects due to stress.

Clonogenic Assay

The colony-forming assay and growth curve analyses were used to assess the sensitivity of the C15.4.3 AP cells to TNFα. Cells were trypsinised, washed, and plated in quintuplicate at a density of 100 per 60-mm Petri dishes. Twenty-four hours after the cells were plated to allow for cell attachment, TNFα was added at concentrations ranging from 19 to 2500 U/ml. Cells were incubated at 37°C in a humidified chamber containing 5% CO2 for 12 days. The colonies were then fixed with a 1:3 (v/v) acetic acid:methanol solution and stained with 10% Giemsa (Sigma Chemical Co., St. Louis, MO); colonies of more than 50 cells were scored. Plating efficiency was calculated with and without TNFα. The dose response
curves were fitted to a four-parameter logistic model, where the response, R, varies with the dose, D, according to the equation: 
\[ R = \frac{a}{1 + \frac{D}{b} c} + R_\infty, \]
and where \( a \) is the difference between the maximum and minimum response, \( b \) is the concentration of drug needed to obtain 50% of the maximal response, \( c \) is a slope factor, and \( R_\infty \) is the maximal effect. The cytotoxic effect of irradiation on asynchronous, exponentially growing C15.4.3 AP cells was also determined by the colony-forming assay. Before irradiation, cell density was determined using appropriate dilutions (100, 300, 600, 1600 cells for 0, 2, 4, and 6 Gy, respectively), and four replicates of each dilution were plated in 60 mm Petri dishes. Cells were irradiated as described above, 24 hours after plating to allow for cell attachment prior to the administration of radiation. The TNFα-containing medium was given at a concentration of 156 U/ml 12 hours before irradiation. A dose of 156 U/ml of TNFα was chosen because colony forming assays showed that this dose was sufficient to induce only partial (50% survival) cell growth when the cytokine was used alone. Cultures were irradiated when the drug was in the medium and were immediately returned to the incubator after irradiation. Colonies were counted after 12 days. Experimentally derived data points are the mean of three experiments. The multi-target model survival curves were fitted to the data using a least squares regression to the linear-quadratic model, \( S = S_0 \exp(-\alpha D_1 - \beta D_1^2), \) where \( D_1 \) is the radiation dose, \( S \) the surviving fraction, and \( S_0 \) a normalizing parameter.

*Tumor growth inhibition* in vivo

All *in vivo* experiments were performed in compliance with the French guidelines for experimental animal studies. CEA transgenic mice were provided by FJ Primus (26).
Two consecutive experiments were performed: Experiment I included the seven different treatment groups (group 1: NaCl, group 2: TNFα, group 3: BsAb, group 4: BsAb+TNFα, group 5: RT, group 6: RT+TNFα, group 7: RT+BsAb+TNFα) and experiment II confirmed the initial results obtained in RT groups.

C15.4.3 AP (1.5 x 10⁶) cells were injected s.c. in the right flank of mice. Tumor-bearing mice were randomized in the different treatment groups when the tumors reached a volume of about 100 mm³. The mice were treated by intravenous (i.v.) injections (200μl) with 0.9% NaCl, TNFα (1 μg/injection), BsAb (25 μg/injection), BsAb+TNFα (ratio 25 μg:1 μg; molar ratio 12.5:1). BsAb-TNFα mixture was prepared 24 hours before injection. Local radiation was performed as described above. On the basis of the biodistribution studies of TNFα and BsAb-TNFα complexes (25), we decided to inject TNFα 3 hours prior to radiation and BsAb-TNFα complexes 24 hours prior radiation. All i.v. injections were performed twice a week for two weeks. Tumor dimensions were measured twice weekly with a caliper and the volumes were calculated by the formula: D1 x D2 x D3 /2 where D1 is the length, D2 the width, and D3 the depth of the tumor. The mice were weighted twice a week and routinely observed for signs of toxicity throughout the study.

mRNA level of TNFα

Following the first week of treatment, tumors were removed and conserved in RNA later stabilization reagent. The mRNA levels of TNFα and of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured by real-time PCR. Total RNA was prepared with the total RNA isolation kit (RNeasy, Qiagen, France) according to the manufacturer’s instructions. The
cDNA was produced from 1 µg of total RNA by reverse transcription with 200 U of Superscript reverse transcriptase (Gibco) in a 20 µl reaction containing 1 x Superscript buffer (Gibco), 1 mM dNTP, 20 ng random hexamer, 10 mM DTT and 20 U of RNase inhibitor. After incubation for 50 min at 42°C, the reaction was terminated by denaturing the enzyme for 10 min at 70°C. RNA integrity was confirmed by denaturing agarose gel electrophoresis and ethidium bromide staining. PCR amplification of TNFα and GAPDH used Syber PCR master Mix; the primer sequences, designed as reported (27) were as follows:

TNFα (forward, 5'-CATCTTCTAAAAATTCGAGTGACAA-3'; reverse, 5'-TGTTGAGTAGCAAGGTACACCC-3');

GAPDH (forward, 5'-TTCACACCACATGGGAAGGC-3'; reverse, 5' -GGCATGGACTGTGGTCATGA-3'). Optimized PCR used the Abi Prism 7700 Sequence detection system (Qiagen). Significant PCR fluorescent signals were normalized to a PCR fluorescent signal obtained from the housekeeping gene GAPDH for each sample.

For expression of the results, the ΔΔCT-method for the relative mRNA quantitation was used. The relative quantitation of target, normalized to an endogenous reference (GAPDH) and a relevant control is given by: relative quantitation = 2-ΔΔCT, with ΔΔCT defined as the difference between the mean ΔCT (treated sample) and the mean ΔCT (untreated sample), ΔCT as the difference between the mean CT (TNFα) and the mean CT (GAPDH as the endogenous control, CT= threshold cycles).

Three consecutive experiments were performed (one mouse per group at each experiment) and mean values with errors were considered for publication.
Histological analyses

Samples of explanted tumors removed after the half delivered treatment, were fixed in the Alcohol-Formalin-Acetic acid (AFA) buffer, and embedded in paraffin. For histopathology examination, sections were stained with haematoxylin and eosin and examined by light microscopy. Semi-quantitative analysis of tumor slides included measurement of necrosis, inflammatory cells, infiltration and necrosis phenomenon.

Two consecutive experiments were performed and mean values were considered for publication.

Statistical analyses

The nonparametric Wilcoxon signed rank test was used to compare the surviving fraction between the two groups (RT alone and RT+TNFα). For in vivo experiments, the results were expressed in terms of the time taken for the tumor to grow after the primary treatment (estimated by the Kaplan-Meier method and compared by the log-rank test), the slope of the tumor repopulation (Anova), and the number of mice cured (Fisher’s exact test) according to treatment groups. All statistical tests were two-sided with an alpha level of 0.05. Data were analysed with software STATA 9.0 (Stata Corporation, College Station, Texas, USA).
RESULTS

In vitro effect of TNFα with or without radiation

The cytotoxic effects of increasing concentrations of TNFα (19 to 2500 U/ml) on asynchronous, exponentially growing C15.4.3 AP cells were determined in colony-forming assays. Cell survival followed a dose-response curve fitted to a four-parameter logistic model as described in Materials and Methods.

Cells were killed by concentrations of TNFα as low as 20 U/ml (Fig. 1A). The LC₅₀, defined as the concentration of drug that reduced the cell survival rate to 50% of that of the controls, was 156 U/ml.

Cell survival following irradiation (Fig. 1B) in aerated medium fitted a linear quadratic model as described in Materials and Methods. D₀ (Dose of radiation giving 37% survival rate) was 1.35 Gy and 6 Gy with or without TNFα (156 U/ml), respectively. The surviving fraction at 2 Gy (SF2) was 0.25 and 0.8 with or without TNFα (156 U/ml), respectively. SF2 was nearly 60% lower in the combined treatment group with significant statistical difference (p < 0.00001). When the data were analyzed according to the linear quadratic model, the α and β components were, respectively, 0.12 Gy⁻¹ and 0.0068 Gy⁻² without TNFα and 0.74 Gy⁻¹ and nearly 0 Gy⁻² with TNFα. These data indicate that treatment with TNFα results in a steeper decline in cell survival due both to a higher initial slope of the dose response curve and a major decrease of the quadratic parameter. These results suggested supra-additivity between the two treatments.
In vivo radiocurability and toxicities with combined treatment

We tested the therapeutic efficacy of the combination of TNFα targeted into tumor by BsAb and radiotherapy in a syngenic model. TNFα was injected i.v. alone or co-injected with the anti-CEA/anti-TNFα BsAb.

Median pre-treatment tumor volumes were 165 (71–220) mm$^3$ without any statistical difference between the groups. In experiment I, no statistical difference in tumor progression was observed after the different primary treatments in the non-irradiated groups (control, TNFα, BsAb, BsAb+TNFα).

In both experiments (I and II) and during a 12-month period of observation, we observed five complete responses out of 10 treated mice (50%), 2/10 (20%), 2/11 (18%), and 0/12 (0%) in the RT+BsAb+TNFα, RT+TNFα, RT alone, and control groups, respectively. This difference was statistically significant when TNFα was targeted with the BsAb as compared with all other irradiated groups ($p = 0.03$). Considering the irradiated groups, the respective slopes of tumor repopulation between day 24 and day 39 (time period around the nadir of the tumor volumes) were 2.6, 2.6, and 1.9 with statistical difference when TNFα was targeted thanks to the BsAb ($p = 0.04$, Fig. 2). With a longer follow-up, the median delay to reach a tumor volume of 1000 mm$^3$ were 80, 72, and 102 days for the RT alone, the RT+TNFα, and the RT+BsAb+TNFα groups, respectively. No statistical difference was observed between the RT and RT+TNFα groups. However, in the presence of the BsAb, borderline significance was obtained ($p = 0.06$) mainly due to the lack of power of the test (in this group, 50% of the mice were cured explaining the low sample size of the remaining mice that recurred).

At the end of all treatments, no significant differences were found in mouse body weight between the seven groups. No diarrhea was observed in any group, suggesting the absence of digestive toxicity. Neither significant fluid retention,
respiratory distress, nor other signs of toxicity were observed in any of the animals during the course of the study.

*Effect of exogenous TNFα on endogenous TNFα mRNA levels.*

After one week of treatment, level of endogenous TNFα mRNA was measured by real-time PCR (Fig. 3). We observed a significant increase in the TNFα mRNA level in the irradiated groups compared to the non-irradiated one. RT alone increased the TNFα mRNA level by a factor of 2.5 ($p < 0.05$) compared to control. TNFα injected i.v. without BsAb enhanced RT-induced TNFα mRNA level in a 2-fold manner ($p < 0.05$). In the RT+BsAb+TNFα group, the level of endogenous TNFα was significantly higher than that observed in RT with or without exogenous free TNFα. Precisely, targeted TNFα with BsAb increased TNFα mRNA by a factor of 6.5 ($p < 0.01$), 2.6 ($p < 0.001$), and 1.3 ($p < 0.01$) compared to controls, RT alone, and RT+TNFα groups, respectively.

*Histological analyses*

After one week of treatment, early cell infiltration and percentage of necrosis were evaluated. Less than 30% necrosis was observed in the control, TNFα, BsAb, BsAb+TNFα, RT, and RT+TNFα groups. In contrast, we observed 90% early necrosis in the BsAb+TNFα+RT group with significant difference as compared with control, RT, or BsAb+TNFα groups (Fig. 4).
DISCUSSION

In 1996, Robert et al. (25) described, for the first time, the use of a BsAb to target a cytokine (TNFα) into a tumor. They demonstrated that the anti-TNFα/anti-CEA BsAb could localize with a high degree of specificity into human colon carcinoma xenografted in nude mice and that the BsAb could help to target to the tumor radiolabeled TNFα either injected 24 or 48 h after the BsAb or co-injected with the BsAb. In the present study, this BsAb was co-injected with TNFα to enhance tumor responses to radiation. In addition, all the experiments were performed in a syngenic model (CEA transgenic mice grafted with CEA transfected cells) to evaluate this therapeutic strategy in a model resembling clinical situation. We have recently shown that TNFα interacts with radiation to enhance killing of some human tumor cell lines in vitro with additive or synergistic effect at a TNFα concentration that produces cytotoxicity in 10% of cells (6–8). We found that TNFα enhanced significantly radiation-induced cell death in several digestive tumor cells, particularly if cells were very sensitive to TNFα used alone (BxPC-3 and CMT93-CEA cells as compared with LS174T cell line). In the present study, C15.4.3AP cells were also found to be highly sensitive in vitro either to TNFα alone or to combined TNFα and RT. The mechanism of interaction between TNFα and RT observed in vitro is not well known but may be related to the production of reactive species induced by each agent (12, 28, 29). This may explain the greater results we obtained in the different sensitive cells to TNFα such as BxPC-3, CMT93-CEA, and C15.4.3AP as compared with LS174T cell line (6–8). In addition, the results of the clonogenic assays in the C15.4.3AP cells confirmed that TNFα is able to switch radiation-induced sublethal lesions to lethal lesions as a “one target-one shot” mechanism. Indeed, in the combined
treatment, the $\alpha$ parameter is 0.74 and the $\beta$ parameter is near to 0 confirming that the surviving fraction decreased as a linear function of the dose (Fig. 1B).

Others mentioned the correlation of the type of TNF receptors (I and II) at the surface of the cells and the cytotoxicity of TNF$\alpha$ (30, 31). We did not find any correlation between the level of TNFR I and II in C15.4.3AP, BxPC-3 (human pancreas cancer), LS174-T (human colon cancer), and CMT-93 (murine colon cancer) cells using a RT-PCR method (data not shown) and the TNF$\alpha$ sensitivity.

In vivo, the effects of TNF$\alpha$ on the immune system have to be considered and may explain the need for an immunocompetent model as described in this article. The transgenic mice express CEA in a specific-tissue manner in humans and allow murine tumor grafts expressing human CEA (26). However, many published data using the nude mice model have shown that TNF$\alpha$ and RT may enhance the therapeutic efficacy of RT in tumor xenograft models (6–11, 32). These results supported the view that the in vivo interactions between TNF$\alpha$ and RT may also be explained by nonimmunological phenomena such as (i) the involvement of endothelial cell integrin $\alpha v\beta 3$ in the disruption of the tumor vasculature by TNF$\alpha$ (3) or (ii) the tumor production of angiostatin following TNF$\alpha$ exposure (33) that could further promote RT local action. Nevertheless, we did not show significant improvements of TNF$\alpha$ and RT combination as compared to RT alone in our syngenic model probably due to a poor fixation to its receptors of free injected TNF$\alpha$. These results reinforce our hypothesis that an anti-CEA/anti-TNF BsAb can concentrate TNF$\alpha$ in a CEA-expressing tumor to improve RT.

The key in vivo results presented in this article are the correlation between the high number of the complete responses, the increased tumor growth delay, the high level of endogenous TNF$\alpha$ mRNA level, and the high percentage of
necrosis (histopathology evaluation) in the RT+BsAb+TNFα group. a. It is clear that exogenous TNFα injected i.v. alone is not sufficient to act as a strong radiosensitizer considering the profile of the tumor growth curves (Fig. 2).

Nevertheless, the role of concentrating TNFα into tumors (25) thanks to our BsAb undoubtedly increased tumor growth delay, intratumoral necrosis, and endogenous TNFα mRNA level. Nevertheless, interpretation of the histopathology data have to take into account that we analyzed more than one section at each experiment but it still only counts as one result.

On one hand, Hallahan et al. showed some time ago an increased TNFα mRNA level after cellular exposure to ionizing radiation (34). On the other hand, Vanhaesebroeck et al. showed a correlation with the expression of the tumor necrosis factor gene in tumor cells and reduced tumorigenicity and invasiveness in vivo (35). In the present study, we showed the superiority of combined treatment (RT+TNFα+BsAb) in endogenous murine TNF mRNA secretion that may explain the high percentage of cured mice never obtained in the xenografted mice (6–8).

Toxicity evaluations were probably underestimated in our model due to the use of recombinant human TNFα which is not recognized by the murine TNFR II. Experiments with murine TNFα using the transgenic mice are in progress allowing a better approach in efficacy and toxicity before starting clinical trials.

Different methods to target TNFα into tumors have recently been published, including intralesional administrations (36). Despite these promising results, we are still convinced that in case of deep tumors such as pancreatic cancers, intravenous injection approaches are less invasive, better accepted by the patient, and easier to perform in daily clinical practice.
In conclusion, our data could be used as a solid preclinical rationale on which to base a clinical study in locally advanced digestive cancers in the near future.
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FIGURE LEGENDS

Fig.1. Dose response curves of the effects of TNFα and irradiation treatment on C15.4.3 AP cells. (A) Response of C15.4.3 AP cells to TNFα. Cells were grown in the presence of increasing concentrations of TNFα (19 to 2500 U/ml). Plating efficiencies were compared with controls grown without TNFα (100% survival). (B) Response of C15.4.3 AP cells to radiation, with or without TNFα. Each point represents the mean +/- SEM of triplicates.

Fig.2. Tumor volume as function of time in the progressing CEA transgenic mice. Seven groups of mice bearing C15.4.3 AP colon carcinoma cell line graft were treated. All groups are detailed on the graphs after removing all cured mice in each group. Values represent mean tumor size of the two consecutive experiments.

Fig.3. mRNA level of murine TNFα. Following the first week of treatment, the tumor of one mouse per group was removed allowing measurement of mRNA levels of endogenous TNFα by real-time PCR. The comparative \( \Delta \Delta C_T \)-method for the relative mRNA quantitation was used for the expression of the results as described in “Materials and Methods”. Three consecutive experiments were performed (one mouse per group at each experiment) and mean values with errors were considered for publication.

Fig.4. Effects of RT and TNFα combination on histologic parameters of explanted C15.4.3 AP tumors. Female transgenic mice with established tumor were treated with (A) vehicle, (B) RT, (C) BsAb + TNFα and (D) BsAb + TNFα +
RT. After the first week of treatment, the tumors were removed and sections of fixed samples embedded in paraffin were stained with haematoxylin and eosin. We then performed two dedicated experiments to histological analyses with one mouse per treatment group.