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The Efficacy of Combined Treatment with Recombinant Human Tumor Necrosis Factor-α and 5-Fluorouracil is Dependent on the Development of Capillaries in Tumor

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Abstract—*The antitumor effects of recombinant human tumor necrosis factor-α (rTNF-α) and 5-fluorouracil (5-FU) in combination treatment were examined on Meth A fibrosarcoma implanted intradermally in mice. Growth of the tumor was inhibited when rTNF-α was given i.v. on day 7 or 11 after implantation, but the effect was countered when 5-FU was additionally given i.p. once a day on days 1–4 after implantation. Conversely, 5-FU given on days 5–8 after implantation augmented the antitumor effects of rTNF-α. Injection of carbon particles showed that fine capillaries did not develop in the tumors of mice treated with 5-FU on days 1–4 after implantation, but that a delicate network of capillaries developed in the tumors of both the mice treated with 5-FU on days 5–8 after implantation and the controls given saline. The results show that the timing of 5-FU treatment is important when attempting to enhance the antitumor effects of rTNF-α, and suggest that these effects are directly associated with newly formed fine capillaries in the tumor.*

INTRODUCTION

TUMOR NECROSIS FACTOR-α (TNF-α) has been found in the serum of mice, rats and rabbits infected with *Bacillus Calmette Guerin* and subsequently challenged with endotoxin [1]. Recombinant human TNF-α (rTNF-α) has been obtained from *Escherichia coli* by a genetic engineering method [2, 3]. rTNF-α causes hemorrhagic tumor necrosis and inhibits the growth of tumors in mice [4–6]. It induces cytotoxic and cytostatic effects against cultured tumor cells *in vitro* [7–9]. The mechanisms by which rTNF-α induces its antitumor effects have been studied. Incubation of endothelial cells with rTNF-α enhances procoagulant activity [10, 11], and induces interleukin 1 (IL-1) [12]. IL-1 interacts with endothelial cells to augment coagulant activity [13, 14]. rTNF-α is cytotoxic for vascular endothelial cells [15] but not for other normal cells [7]. Thus, one of the targets of rTNF-α seems to be the vascular endothelial cells.

The antitumor effects of rTNF-α have been enhanced by such chemotherapeutic drugs as Adriamycin®, etoposide (VP16), 5-fluorouracil (5-FU) and cyclophosphamide [16, 17], immunomodulators [18–22], and hyperthermia [23, 24] in *in vivo* and *in vitro* experimental models. However, cyclooxygenase inhibitors were found to alleviate some of the toxicity of TNF-α for the host [25, 26]. Recently, we showed that serotonin receptor blockers inhibit the antitumor effects of rTNF-α in mice, and suggested that serotonin is an intermediate [27]. The study of combination therapy of rTNF-α with other drugs would provide more information on the mechanism of rTNF-α action.

In this paper, we studied the combined antitumor effects of rTNF-α and 5-fluorouracil (5-FU) in mice, and found that the efficacy of rTNF-α therapy depends upon the timing of injection of 5-FU. The relationship between the development of capillaries in tumors after the injection of 5-FU and rTNF-α therapy is discussed in this context.

MATERIALS AND METHODS

Animals and tumor

Female BALB/c mice (7–9 weeks old), purchased from Charles River Japan Inc., Atsugi, Japan, were

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used in groups of five to 10. Meth A fibrosarcoma (Meth A) was maintained i.p. by serial passage in BALB/c mice.

Drugs

rTNF-α, supplied by Genentech Inc., South San Francisco, CA, was diluted in phosphate-buffered saline (PBS, 10 mM, pH 7.0) just before use. The purity was more than 99.0% when tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. rTNF-α had a specific activity of approximately 4.1×10^7 units/mg protein, which was expressed as the dilution point for 50% cytotoxicity against actinomycin D-treated L929 cells. 5-FU, purchased from Kyowa Hakko Co. Ltd. (Tokyo, Japan), was dissolved in and diluted with saline. rTNF-α and 5-FU were given in volumes of 0.2 ml/mouse and 10 ml/kg, respectively.

Evaluation of antitumor effects

Meth A was implanted intradermally (i.d.) into the left flank of BALB/c mice. Drug efficacy against Meth A was expressed as mean tumor weight. Tumor weight, as derived from caliper measurements of length (a) and width (b) of the tumor in mm, and length (an) and width (bn) of tumor necrosis in mm, was calculated by the formula described by Haranaka *et al.* [28], where tumor weight (mg) = $1/2 \times a \times b^2 - 1/2 \times an \times bn^2$.

Light microscopy

To study the development of capillaries in the tumor, carbon particle solution (drawing ink, Rotringwerke, Hamburg, F.R.G., average particle size about 160 μm) suspended 1:5 in saline containing 1% gelatin (Nakarai Chemicals, Ltd., Kyoto, Japan) was given i.v. to mice 5 min before removal of the tumor. The tumor was fixed with 10% buffered formaldehyde and frozen. Sections were made at 50 μm and observed under microscopy.

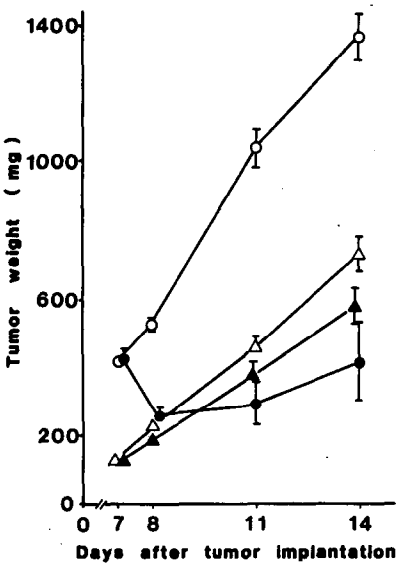

Fig. 1. Inhibition of the antitumor effect of rTNF-α by 5-FU in mice. Meth A cells (3×10^6) were implanted i.d. in mice on day 0. On days 1–4, 5-FU 32 mg/kg or saline was given i.p. once a day, and on day 7 rTNF-α 1 μg/mouse was given i.v. Tumor weight was measured; values are mean ± S.E. Each group consisted of 10 mice. ○: Saline (control), ●: rTNF-α, △: saline in 5-FU treated mice, ▲: rTNF-α in 5-FU-treated mice.

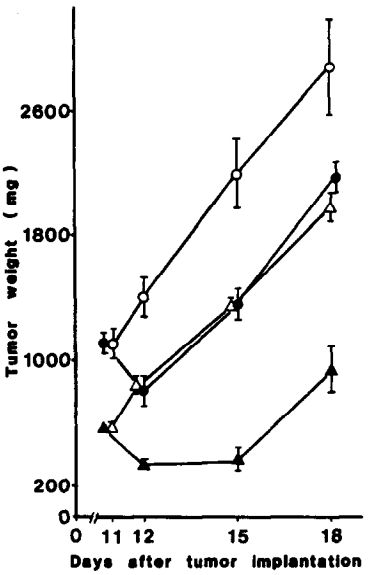

Fig. 2. Enhancement of the antitumor effect of rTNF-α by 5-FU in mice. Meth A cells (3×10^6) were implanted i.d. in mice on day 0. On days 5–8, 5-FU 32 mg/kg or saline was given i.p. once a day, and on day 11 rTNF-α 1 μg/mouse was given i.v. Tumor weight was measured; values are mean ± S.E. Each group consisted of 10 mice. ○: Saline (control), ●: rTNF-α, △: saline in 5-FU treated mice, ▲: rTNF-α in 5-FU treated mice.

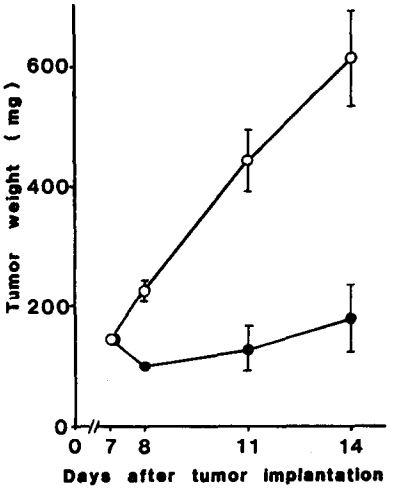

Fig. 3. Antitumor effect of rTNF-α on small tumors in mice. Meth A cells (3×10^5) were implanted i.d. in mice on day 0. On day 7, rTNF-α 1 μg/mouse was given i.v. Tumor weight was measured; values are mean ± S.E. Each group consisted of 10 mice. ○: Saline (control), ●: rTNF-α.

RESULTS

Effect of 5-FU on the antitumor activity of rTNF-α

Meth A cells (3×10^6) were implanted i.d. into mice on day 0. On days 1–4, 5-FU 32 mg/kg or

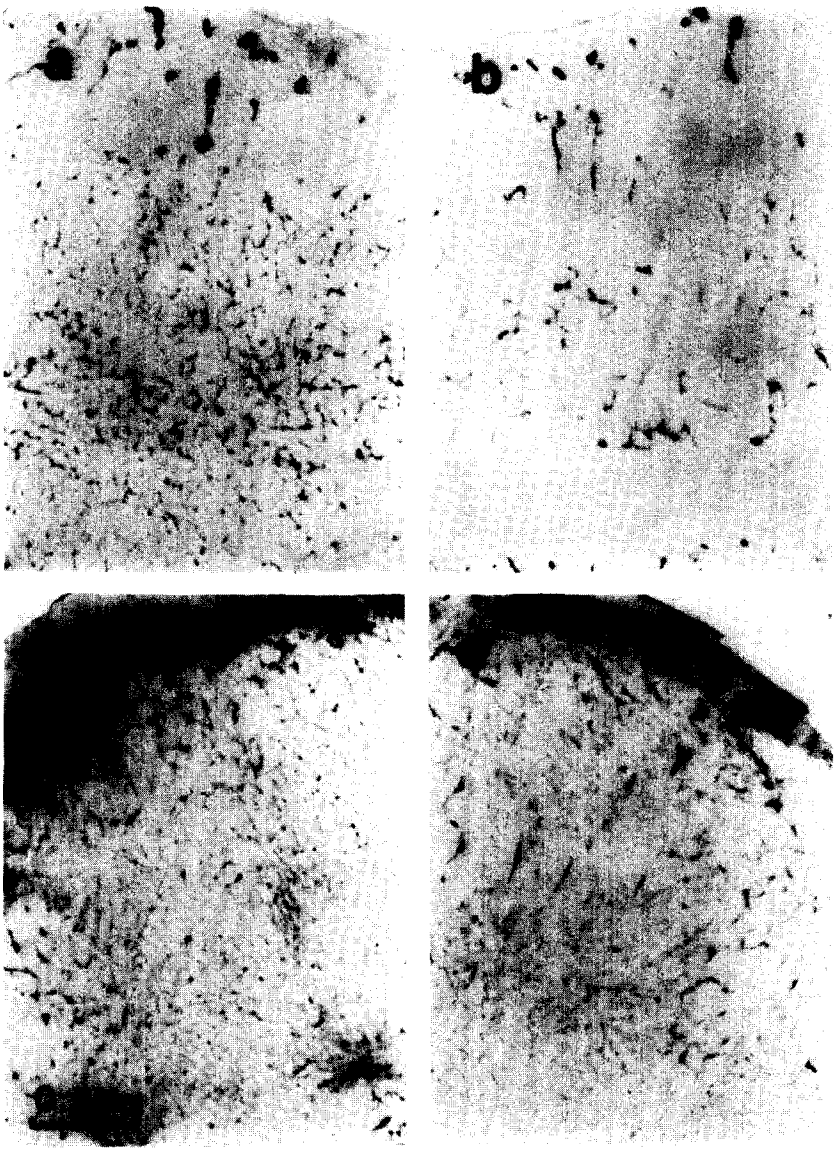

Fig. 4. *Distribution of carbon particles in newly formed capillaries in tumor. Meth A cells* (3×10^6) *were implanted i.d. in mice on day* 0. *Saline* (*a*) *or* 5-*FU* 32 mg/kg (*b*) *was given i.p. on days* 1–4, *and on day* 7 *carbon particles were given i.v. In the other experiment, saline* (*c*) *or* 5-*FU* 32 mg/kg (*d*) *was given i.p. on days* 5–8, *and on day* 11 *carbon particles were given i.v. Each group consisted of five mice.*

saline was given i.p. once a day, and the tumors were smaller in the 5-FU-treated mice on day 7 than in the saline-treated mice. When rTNF-α 1 μg/mouse was given i.v. on day 7, it inhibited the growth of tumor in the saline-treated mice, but not in the 5-FU-treated mice (Fig. 1).

In the other experiment, 5-FU 32 mg/kg or saline was given i.p. once a day on days 5–8 to mice bearing Meth A, and the tumors were smaller in the 5-FU-treated mice on day 11 than in the saline-treated mice. On day 11, rTNF-α 1 μg/mouse was given i.v. to the mice treated with 5-FU or saline, and rTNF-α markedly inhibited the growth of tumor in both groups of animals (Fig. 2). Tumor weight after injection of rTNF-α in mice treated with 5-FU was the smallest among the groups.

Antitumor effects of rTNF-α on Meth A of small size

To study whether the preventive effect of 5-FU on the antitumor effects of rTNF-α derives from mechanisms related to the size of tumors, a small number of Meth A cells (3×10^5) were implanted i.d. in mice on day 0. When rTNF-α 1 μg/mouse was given i.v. on day 7, it markedly inhibited tumor growth in mice (Fig. 3). The tumor (Fig. 3) on day 7 in mice implanted with a small number of cells (3×10^5) was almost the same as that in the mice implanted with a large number of cells (3×10^6) and treated with 5-FU from days 1–4 (Fig. 1).

Development of capillaries

We stained blood vessels by i.v. injection of carbon particles in mice, and examined the development of new capillaries in the tumor. On day 7 after the implantation of Meth A cells (3×10^6), the capillaries in tumors of mice treated with 5-FU 32 mg/kg from days 1–4 were much less extensively developed and finer than those in mice treated with saline (Fig. 4a and b). On day 11, a delicate network of fine capillaries formed in both the mice treated with saline and those treated with 5-FU 32 mg/kg from days 5–8 (Fig. 4c and d).

DISCUSSION

In this study, when 5-FU was given i.p. to mice on days 1–4 after the implantation of Meth A, the antitumor effect of rTNF-α was inhibited. However, in mice treated with 5-FU on days 5–8, a strong synergism between rTNF-α and 5-FU enhanced their antitumor effects. The results suggest that the timing of the injection of 5-FU is crucial in rTNF-α therapy.

Antitumor chemotherapeutics with cytotoxic activity are known to be more effective on small solid tumors than on large ones. However, we previously showed that rTNF-α had no cytotoxicity against cultured Meth A cells *in vitro*, and that it was more effective on large solid tumors on day 7 after tumor implantation than on small ones on day 3 [6, 29]. Similarly Mulé *et al.* [30] recently reported that, in mice, small tumors were much less sensitive to rTNF-α than large ones. In this study, we showed that the tumor size was smaller in mice treated with 5-FU from days 1–4 after implantation than in the saline-treated group (Fig. 1). It is possible that rTNF-α has no or slight effect on small tumors. Thus, we studied the relation of tumor size to rTNF-α therapy, and found that rTNF-α induced antitumor effects when given on day 7 after the implantation of small number of Meth A cells (3×10^5) in mice (Fig. 3). The tumor, just before the injection of rTNF-α (on day 7), was as small as that in mice treated with 5-FU from days 1–4 after implantation of 3×10^6 cells (Fig. 1). These results suggest that the antitumor effects of rTNF-α are not dependent on tumor size in mice treated previously with 5-FU.

Why should the time of injection of 5-FU have such an effect on the response to rTNF-α? In our previous paper, we suggested that rTNF-α selectively impairs the microcirculation in newly formed capillaries in tumors, and leads to the lysis of tumor cells *in vivo* [29]. Recently, Nawroth *et al.* [31] also reported that TNF leads to localized fibrin deposition with the formation of occlusive intravascular thrombi in tumor capillaries. Furthermore, Proietti *et al.* [32] showed that TNF induces marked vascular congestion in tumor blood vessels. These findings suggest that thrombus formation in newly formed capillaries of tumor tissue is important for the induction of antitumor effects of rTNF-α. Thus, we stained blood vessels by the injection of carbon particles to study the relationship between the development of capillaries in tumors treated with 5-FU and the antitumor effect of rTNF-α. Treatment with 5-FU from days 1–4 impaired the development of fine capillaries in the tumors, while a delicate network of capillaries formed in the saline-treated mice. Treatment with 5-FU on days 5–8 did not affect the network of capillaries in tumor. The results suggest that the capillaries are established within 5 days after tumor implantation, and that 5-FU has no effects on the established capillaries.

In the present results, the antitumor effect of rTNF-α was countered by 5-FU when the tumor capillaries were not fully developed, but the effect of rTNF-α was enhanced when a delicate network of capillaries had formed in the tumor. Similar results were observed in Meth A-bearing mice treated with the combination of rTNF-α and cyclophosphamide (data not shown). Thus, we suggest that the *in vivo* antitumor effects of the combined treatment with rTNF-α and 5-FU depend upon the development of capillaries in tumors. These findings could give more information on combination treatment with rTNF-α and chemotherapy in patients.

REFERENCES

1. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA* 1975, **72**, 3666–3670.
2. Aggarwal BB, Kohn WJ, Hass PE *et al.* Human tumor necrosis factor: production, purification and characterization. *J Biol Chem* 1985, **260**, 2345–2354.
3. Pennica D, Nedwin GE, Hayflick JS *et al.* Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* 1984, **312**, 724–729.
4. Balkwill FR, Lee A, Aldam G *et al.* Human tumor xenografts treated with recombinant human tumor necrosis factor alone or in combination with interferons. *Cancer Res* 1986, **46**, 3990–3993.
5. Sohmura Y, Nakata K, Yoshida H, Kashimoto S, Matsui Y, Furuichi H. Recombinant human tumor necrosis factor—II. Antitumor effect on murine and human tumors transplanted in mice. *Int J Immunopharmacol* 1986, **8**, 357–368.
6. Manda T, Shimomura K, Mukumoto S *et al.* Recombinant human tumor necrosis factor-α: evidence of an indirect mode of antitumor activity. *Cancer Res* 1987, **47**, 3707–3711.
7. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA Jr, Shepard HM. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. *Science* 1985, **230**, 943–945.
8. Nakano K, Abe S, Sohmura Y. Recombinant human tumor necrosis factor—I. Cytotoxic activity *in vitro*. *Int J Immunopharmacol* 1986, **8**, 347–355.
9. Fransen L, Van der Heyden J, Ruysschaert R, Fiers W. Recombinant tumor necrosis factor: its effect and its synergism with interferon-γ on a variety of normal and transformed human and mouse cell lines. *Eur J Cancer Clin Oncol* 1986, **22**, 419–426.
10. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc Natl Acad Sci USA* 1986, **83**, 4533–4537.
11. Nawroth P, Stern D. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J Exp Med* 1986, **163**, 740–745.
12. Nawroth P, Bank I, Handley D, Cassimeris J, Chess L, Stern D. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J Exp Med* 1986, **163**, 1363–1375.
13. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA Jr. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J Exp Med* 1984, **160**, 618–623.
14. Nachman RL, Hajjar KA, Silverstein RL, Dinarello CA. Interleukin 1 induces endothelial cell synthesis of plasminogen activator inhibitor. *J Exp Med* 1986, **163**, 1595–1600.
15. Sato N, Goto T, Haranaka K *et al.* Actions of tumor necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition, and cytotoxicity. *J Natl Cancer Inst* 1986, **76**, 1113–1121.
16. Alexander RB, Nelson WG, Coffey DS. Synergistic enhancement by tumor necrosis factor of *in vitro* cytotoxicity from chemotherapeutic drugs targeted at DNA topoisomerase II. *Cancer Res* 1987, **47**, 2403–2406.
17. Regenass U, Müller M, Curshellas E, Matter A. Anti-tumor effects of tumor necrosis factor in combination with chemotherapeutic agents. *Int J Cancer* 1987, **39**, 266–273.
18. Chen L, Suzuki Y, Wheelock EF. Interferon-gamma synergizes with tumor necrosis factor and with interleukin 1 and requires the presence of both monokines to induce antitumor cytotoxic activity in macrophages. *J Immunol* 1987, **139**, 4096–4101.
19. Hori K, Ehrke MJ, Mace K, Mihich E. Effect of recombinant tumor necrosis factor on tumoricidal activation of murine macrophages: synergism between tumor necrosis factor and gamma-interferon. *Cancer Res* 1987, **47**, 5868–5874.
20. Nishimura T, Ohta S, Sato N, Togashi Y, Goto M, Hashimoto Y. Combination tumor-immunotherapy with recombinant tumor necrosis factor and recombinant interleukin 2 in mice. *Int J Cancer* 1987, **40**, 255–261.
21. Ruggiero V, Baglioni C. Synergistic anti-proliferative activity of interleukin 1 and tumor necrosis factor. *J Immunol* 1987, **138**, 661–663.
22. Talmadge JE, Tribble HR, Pennington RW, Phillips H, Wiltrout RH. Immunomodulatory and immunotherapeutic properties of recombinant gamma-interferon and recombinant tumor necrosis factor in mice. *Cancer Res* 1987, **47**, 2563–2570.
23. Watanabe N, Niitsu Y, Umeno H *et al.* Synergistic cytotoxic and antitumor effects of recombinant human tumor necrosis factor and hyperthermia. *Cancer Res* 1988, **48**, 650–653.
24. Niitsu Y, Watanabe N, Umeno H *et al.* Synergistic effects of recombinant human tumor necrosis factor and hyperthermia on *in vitro* cytotoxicity and artificial metastasis. *Cancer Res* 1988, **48**, 654–657.
25. Kettelhut IC, Fiers W, Goldberg AL. The toxic effects of tumor necrosis factor *in vivo* and their prevention by cyclooxygenase inhibitors. *Proc Natl Acad Sci USA* 1987, **84**, 4273–4277.
26. Talmadge JE, Bowersox O, Tribble H, Lee SH, Shepard HM, Liggitt D. Toxicity of tumor necrosis factor is synergistic with gamma-interferon and can be reduced with cyclooxygenase

inhibitors. *Am J Pathol* 1987, **128**, 410–425.
27. Manda T, Nishigaki F, Mori J, Shimomura K. Important role of serotonin in the antitumor effects of recombinant human tumor necrosis factor-α in mice. *Cancer Res* 1988, **48**, 4250–4255.
28. Haranaka K, Satomi N, Sakurai A. Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice. *Int J Cancer* 1984, **34**, 263–267.
29. Shimomura K, Manda T, Mukumoto S, Kobayashi K, Nakano K, Mori J. Recombinant human tumor necrosis factor-α: thrombus formation is a cause of antitumor activity. *Int J Cancer* 1988, **41**, 243–247.
30. Mulé JJ, Asher A, McIntosh J *et al.* Antitumor effect of recombinant tumor necrosis factor-α against murine sarcomas at visceral sites: tumor size influences the response to therapy. *Cancer Immunol Immunother* 1988, **26**, 202–208.
31. Nawroth P, Handley D, Matsueda G *et al.* Tumor necrosis factor/cachectin-induced intravascular fibrin formation in meth A fibrosarcomas. *J Exp Med* 1988, **168**, 637–647.
32. Proietti E, Belardelli F, Carpinelli G *et al.* Tumor necrosis factor-α induces early morphologic and metabolic alterations in friend leukemia cell tumors and fibrosarcomas in mice. *Int J Cancer* 1988, **42**, 582–591.