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In Vitro Antiproliferative Efficacy of Interferon-Alpha, -Gamma and Tumor Necrosis Factor on Two Human Renal Tumor Xenografts

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Summary. The in vitro antitumor activity of recombinant alpha- and gamma-Interferon as well as recombinant Tumor Necrosis Factor alpha was tested on renal cell carcinoma xenografts using the double layer soft agar method. Using this assay, the effect of the drugs on the clonogenic potential of tumor cells in soft agar was determined and used as an indication for the antiproliferative capacity of these drugs. There appeared to be a differential response of the tested xenografts towards these drugs in a dose dependent way. However, when used in combination in most cases an additive or synergistic effect was observed. Even though again the response was differential, the combination of alpha-Interferon (10 ng/dish) and Tumor Necrosis Factor (100 ng/dish) resulted in a complete inhibition of colony formation in soft agar for both tumor lines. We conclude that there is a direct effect of alpha-Interferon, gamma-Interferon and Tumor Necrosis Factor on renal cell carcinoma xenografts. In combination drug tests the effect of alpha-Interferon and Tumor Necrosis Factor was strongly synergistic. The implication of these studies for in vivo use of these drugs remain to be established.

Key words: Interferon – Tumor Necrosis Factor – Renal cell tumor – Xenograft – Soft agar

Introduction

For patients with renal cell carcinoma (RCC) the 5-year survival rate has not significantly improved during the last two decades [29]. New therapeutic regiments against RCC are difficult to obtain because patients present with the disease in a wide range of tumor stage. Transplantation of human renal cell carcinoma into athymic nude mice, however, provided experimental tumor models which make the development of new therapeutic approaches possible [25, 36]. Therapeutic regimens using Biological Response Modifiers (BRM's) appear to be very promising as anticancer therapy in experimental animals and in man. Interferons appear to have a dual mode of effect; directly on the cancer cell and indirectly through the immune system. Direct effects on tumor cells were shown in a tumor model system of heterotransplantion in nude mice of breast tumors [3], fibrosarcoma [24] and bowel carcinoma [4]. Indirect effects were observed when murine Interferon (IFN) was used in the treatment of human tumor xenografts in the nude mouse host [2, 5], which indicates that murine IFN can mediate tumor inhibition via an immunological effect in the T-cell deficient host.

The combined direct and indirect in vitro antiproliferate effects of rIFN-gamma to renal cells were tested by Saito et al. [42] using a clonogenic assay with human adherent ascites cells which contained $>80\%$ macrophages as feeder cells in a separate agarose underlayer. When treated with human rIFN-gamma these cells became strongly inhibitory to tumor colony growth. Hence it seems that human tumor associated macrophages may become tumoricidal under the influence of rIFN-gamma producing a diffusable substance in agarose culture which caused the observed antiproliferative effects on tumor cells.

Host mediated antitumor effects of Interferons were already suggested by Belardelli et al. [6, 7] and Pace et al. [38] and since the marcrophage is the cellular source of Tumor Necrosis Factor (TFN) [28], much attention has been payed to this antitumor agent [34, 35]. TNF has direct in vitro cytotoxic activity against tumor cells [19, 45] but TNF also induces a host mediated factor which contributes to the antitumor effects, especially in relation to T-cells [17, 20]. The synergistic antitumor activity of IFN-gamma and TNF is now well documented for in vitro [14, 15, 31, 41, 43, 50] and in vivo systems [8]. Williamson et al. [49] also found a synergistic antitumor effect of IFN-alpha and TNF on human breast and colon cancer cells.

Since IFN-alpha appears to be potential active antitumor agent in patients with renal cell carcinoma [9, 32, 33, 39] and TNF also shows in vitro cytostatic and cytolytic effects on human renal cell carcinoma cell lines [21] we decided to

test the direct in vitro antiproliferative effects of human recombinant alpha- and gamma-IFN as well as human recombinant TNF on two renal cell carcinoma tumor lines; NC-65 [23] and RC-43 [26].

In this study we showed that alpha- and gamma-IFN as well as TNF have in vitro antiproliferative effects on RCC tumor cells.

Material and Methods

Renal Cell Carcinoma Xenografts

After original subcutaneous transplantation of small tumor pieces (2 mm^3) in both flanks of Balb-C nu/nu mice, the RC-43 and NC-65 xenografts were passaged every four weeks. Tumor material for testing was harvested after four weeks.

Biological Respons Modifiers

Human IFN-alpha, IFN-gamma and TNF-alpha, obtained from Boehringer Ingelheim, were produced in *E. coli* by recombinant DNA technology. The specific activity of IFN-alpha and IFN-gamma was 3.2E8 units/mg protein and 2E7 units/mg protein respectively. It was measured by inhibition of encephalomyocarditis (EMC) virus replication in A549 cells with reference to the National Institute of Health (NIH) leucocyte IFN-alpha standard Go 23-901-527 and the NIH IFN-gamma standard Gg 23-901-530. The purity of both IFN's was > 98% as determined by SDS polyacrylamide gel electrophoresis and the amount of endotoxin was less than 1.0 ng/mg protein for IFN-alpha and less or equal to 0.5 ng/mg protein for IFN-gamma as based on the limulus amoebocyte lysate assay. The specific activity of TNF-alpha determined in the presence of actinomycine-D was 6E7 units/mg protein as determined in the L-929 cytotoxicity assay. The purity was > 99% as determined by SDS polyacrylamide gel electrophoresis and it contained 1.0 ng or less endotoxin/mg protein based on the limulus amoebocyte lysate assay. The drugs were dissolved in the accessory dissolvent and diluted with double enriched (D.E.) CMRL 1066 medium (Gibco, Paisley, UK).

Preparation of Single Cell Suspensions

When tumors reached sizes of 1.5 x 1.5 cm the mice were sacrificed and tumor material was suspended in McCoy's wash (Gibco, Paisley, UK). After careful removal of areas of tumor necrosis, tumors were cut into pieces of about 3 to 4 mm^3. The tumor pieces were then minced with scissors into a 300 μ metal sieve and continuously washed with McCoy's solution into a petri dish. The minced tumor tissue was passed twice through a 40–70 μ nylon filter (Ortho Diagnostics, Beerse, Belgium) to obtain a single cell suspension. The cells were centrifuged at room temperature at 400 g for 5 minutes after which the supernatant was discarded. Upon resuspension of the cell pellet in double enriched CMRL 1066, cell density and viability were determined by adding 15 μl trypan blue solution (25 mg in 5 ml 3% acetic acid) to 15 μl cell suspension and simultaneously counting coloured and not coloured cells using a Bürker Türk haemocytometer.

Human Tumor Colony Forming Assay (HTCS)

For the detection of the growth potential of tumor cells in soft agar a modified two layer soft agar culture method as originally described by Hamburger and Salmon [18] was used [47]. Tumor cell suspensions were plated at a concentration of 10E5 tumor cells per dish in the upper layer of the two-layer agar culture system. The cells were cultured immediately after preparation of the single cell suspension and growth potential was quantified using an Omnicon Fas II automated colony counter (Milton Roy Inc., Rochester, New York, USA) [22]. For dynamic colony growth development we used the "Temporal Growth Pattern" method giving an estimation of growth over a certain period of time [11].

Drug Testing

Drug tests were performed as continuous exposure of the tumor cells to the drugs. The drugs were layered over the top agar in a volume of 200 μl D.E. CMRL-1066 medium. The dishes in the growth control were layered with 200 μl D.E. CMRL-1066 and as a cytotoxic growth control we used a 0.37 mM HgCl2 solution.

Results

Effect of IFN-Alpha, IFN-Gamma and TNF on the Colony Forming Capacity of the Human Tumor Xenografts RC-43 and NC-65

After determination of optimal plating densities which apeared to be 10E5 cells per dish for both tumor lines (data not shown), single drug tests were performed using IFN-alpha, IFN-gamma and TNF at concentrations of 10, 100 and 1,000 ng/dish (= ng/2 ml). Figure 1A and B show

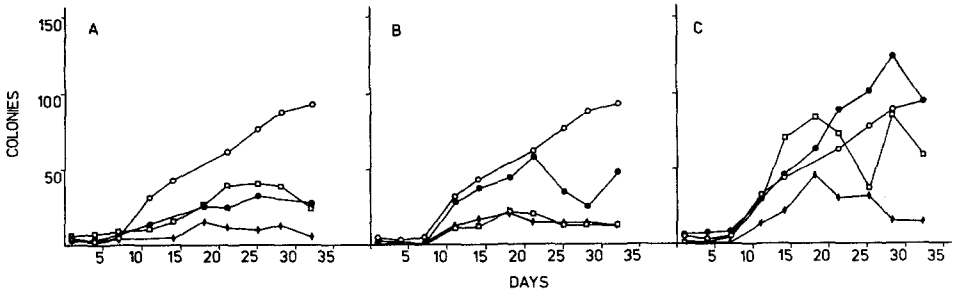

Fig. 1A–C. Effect of three different Biological Respons Modifiers on colony formation of RC-43 renal tumor xenograft cells in soft agar after application of a single dose of the drugs. **A** IFN-alpha. Specific activity: 3.2.10E8 IU/mg. **B** IFN-gamma. Specific activity: 2.010E7 IU/mg. **C** TNF. Specific activity: 6.0.10E7 IU/mg. ○—○ = Growth control, ●—● = BRM concentration 10 ng/dish, □—□ = BRM concentration 100 ng/dish, ◇—◇ = BRM concentration 1,000 ng/dish

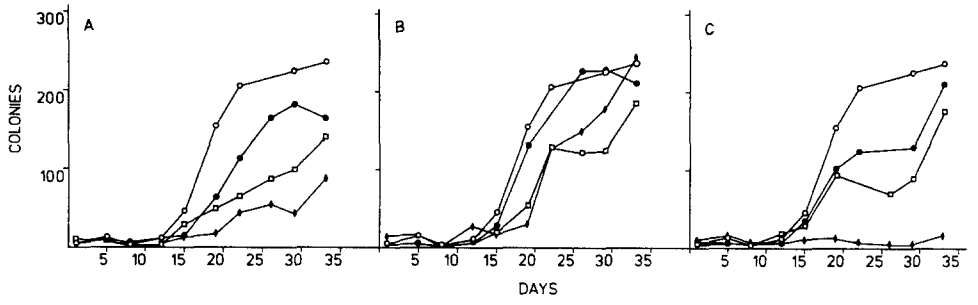

Fig. 2A–C. Effect of three different Biological Respons Modifiers on colony formation of NC-65 renal tumor xenograft cells in soft agar after application of a single dose of the drugs. **A** IFN-alpha. Specific activity: 3.2.10E8 IU/mg. **B** IFN-gamma. Specific activity: 2.0.10E7 IU/mg. **C** TNF. Specific activity: 6.0.10E7 IU/mg. o—o = Growth control, •—• = BRM concentration 10 ng/dish, □—□ = BRM concentration 100 ng/dish, ◇—◇ = BRM concentration 1,000 ng/dish

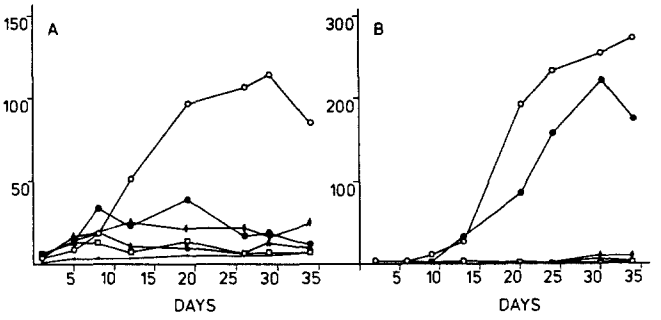

Fig. 3A and B. Effect of combinations of IFN-alpha (10 ng/dish), IFN-gamma (10 ng/dish) and TNF (100 ng/dish) on colony formation of **A** RC-43 and **B** NC-65 renal tumor xenograft cells in soft agar after application of a single dose of the drugs. The combination IFN-alpha/IFN-gamma/TNF in B. coincides with the IFN-alpha/TNF combination. o—o = Growth control, •—• = cytotoxic growth control (200 μg HgCl2/dish), •—• = IFN-alpha, IFN-gamma, □—□ = IFN-alpha, TNF, ◇—◇ = IFN-gamma, TNF, △—△ = IFN-alpha, IFN-gamma, TNF

that both IFN-alpha and IFN-gamma inhibited colony formation of RC-43 cells in vitro. Although a dose dependent inhibition could not be shown, an IFN dose of 1,000 ng/dish inhibited colony formation stronger than a dose of 10 ng/dish. However, for RC-43 a dose dependent colony forming inhibition after TNF administration was evident.

A dose dependent in vitro colony forming inhibition of IFN-alpha and TNF on NC-65 renal tumor cells was found, which is illustrated in Fig. 2A and C respectively. The number of colonies formed using 1,000 ng/dish of TNF did not exceed the number of colonies in the HgCl2 cytotoxic growth control. Gamma-IFN on the other hand, appeared to have very little effect on colony formation of these cells in soft agar. After a delayed formation of colonies from about the second week of the experiment, final colony formation was completed at the growth control level.

Synergistic Effects of Combinations of IFN-Alpha, IFN-Gamma and TNF on the Colony Forming Capacity of RC-43 and NC-65 Cells

By using drugs in the lowest possible concentrations on the assumption that IFN and TNF can act synergistically [30, 50], combination drug tests were performed with both Interferons and with TNF. Interferon concentrations of 10 ng/dish were used because at this concentration colony forming inhibition has been shown for both alpha- and gamma-IFN and the TNF concentration used in these tests was 100 ng/dish because this concentration showed colony forming inhibition for both tumor lines. Since the TNF concentration of 10 ng/dish seemed to stimulate the colony formation on the RC-43 line as shown in Fig. 1C this concentration was not chosen.

The results of the combination tests are shown in Fig. 3A and 3B and are summarized in Table 1 which shows the percentage survival values for the combination drug tests as well as those for the single drug tests.

Figure 3A shows the in vitro colony forming capacity of RC-43 renal carcinoma cells when treated with different combinations of two of the above mentioned BRM's. Calculated according to Valeriote and Lin [46], synergistic effects were found for all different combinations on RC-43. The number of colonies in the alpha-gamma-IFN combination rising hardly above HgCl2 cytotoxic control level. The most effective combination appears to be that of alpha-IFN with TNF in which colony formation equals that of the cytotoxic control. The combination of gamma-IFN with TNF, however, proves to be less effective for this tumor since it gives rise to about 20% survival as compared to the growth control.

Figure 3B, in which the in vitro colony formation of NC-65 renal carcinoma cells is shown after treatment with different combinations of the tested BRM's, shows a rather high survival percentage of these cells with the drug combination of alpha-IFN and gamma-IFN as compared to the RC-43 cells. This combination results in an antitumor effect which according to Valeriote and Lin is sub-additive because the survival fraction for these cells with this drug combination had to be less than 63% to be classified as synergistic. The most effective treatment of these tumor cells, however, also appears to be the combination of alpha-IFN and TNF. Furthermore the drug combination of gamma-IFN and TNF appears to have a similar effect because the number of formed colonies is only 2% of the number of colonies formed in the growth control.

Table 1. Percentage survival after drug therapy. Percentage survival of RC-43 and NC-65 renal tumor xenograft cells after growth in soft agar and application of a single dose of different combinations of IFN-alpha, IFN-gamma and TNF as compared to growth in the untreated control. Concentrations are given in ng/dish (ng/2 ml)

Drug combinations	% survival RC-43	% survival NC-65
α-IFN 10	25	70
γ-IFN 10	45	90
TNF 100	55	75
α-IFN 10, γ-IFN 10	3	65
α-IFN 10, TNF 100	0	0
γ-IFN 10, TNF 100	20	2
α-IFN 10, γ-IFN 10, TNF 100	1	0

Table 1 summarizes these results. Also, given in this table are the results of an experiment in which a triple combination of alpha-IFN, gamma-IFN and TNF is tested on both tumor lines. With this combination the percentage survival values of 1 and 0% for RC-43 and NC-65 respectively confirm the results of the tests with a combination of two drugs, with the value of 1% survival for RC-43 laying within experimental spread.

Discussion

Experimental results presented in this paper show that except for the effect of gamma-IFN on NC-65 tumor cells, a dose dependent inhibition of the colony forming capacity in soft agar was evident for RC-43 and NC-65 renal tumor cells when treated with IFN-alpha, IFN-gamma and TNF in direct exhibition of the tumor cells to these drugs. There appeared to be a difference in sensitivity towards IFN-alpha between the two tested renal tumors (Figs. 1A, 2A). Whereas RC-43 is very sensitive for this lymphokine (percentage survival was only 25% of growth control with an IFN-alpha concentration of 10 ng/dish), NC-65 appeared to be moderately sensitive (percentage survival about 70% of growth control at 10 ng/dish). Hence, the dose dependency for this drug was more distinct for this tumor than for the RC-43 tumor.

Figures 1B and 2B show that in vitro sensitivity for gamma-IFN was more profound for RC-43 tumor cells than for NC-65 tumor cells. The percentage survival for RC-43 cells at a gamma-IFN concentration of 10 ng/dish was about 45% of growth control level. NC-65, however, showed very little in vitro sensitivity for this BRM.

In vitro colony growth inhibition of TNF on the two renal cell carcinomas appeared to be somewhat different from the effect of IFN-alpha and IFN-gamma on colony formation of these lines. Sensitivity of RC-43 towards the tested Interferons appeared to be greater than the sensitivity of NC-65 cells towards these BRM's. TNF sensitivity, however, was greater for NC-65 cells than for RC-43 cells (Figs. 1C and 2C) which shows that RC-43 and NC-65 tumor cells responded differentially to this single drug treatment. Complete inhibition of colony formation in soft agar was shown for NC-65 cells when treated with 1,000 ng TNF/dish, whereas the percentage survival for RC-43 tumor cells at this concentration was still 15% of growth control level. A very interesting difference in the sensitivity of both tumor lines towards TNF, however, was the slight apparent stimulation of colony formation of RC-43 cells when treated with 10 ng TNF/dish. Even with a TNF concentration of 100 ng/dish a stimulation of colony formation was measured around the second week of the experiment. After further treatment, however, the number of colonies formed in the growth control rose above the 100 ng/dish level which ended with a percentage survival of about 55% of growth control level after 4.5 weeks of growth. A well documented effect of TNF was the in vitro stimulation of fibroblast growth [44, 48] but whether low concentrations of TNF could display such an effect on tumor cells was not known.

The observed growth patterns in the combination tests showed that most of the combinations resulted in a strong inhibition of colony formation. The most effective drug combination for both tumor lines was the combination alpha-IFN 10 ng/dish and TNF 100 ng/dish which resulted in a percentage survival of 0% as compared to the growth control which was a strong synergistic effect with respect to the single drug tests. Another synergistic effect was shown by the combination of alpha- and gamma-IFN when administered to RC-43 tumor cells. This combination resulted in a percentage survival of only 3% of control level, whereas percentage survival appeared to be 25 and 45% for alpha-IFN and gamma-IFN respectively when administered as single drugs. For NC-65 cells, however, only sub-additive effects were produced by this drug combination. Synergistic effects occurred with the combination IFN-gamma 10 ng/dish and TNF 100 ng/dish when administered to RC-43 tumor cells. The synergistic anti-tumor effect of this combination on NC-65 cells, was more marked. The surviving fraction was only 2% of control level indicating that the effect was highly synergistic since the values for the single drugs were 90% and 75% for gamma-IFN and TNF respectively.

Because Vinblastine seems to be a promising agent in the treatment of renal cell carcinoma [37], and combinations of alpha- and gamma-IFN with Vinblastine showed synergistic effects against renal cell carcinoma in vitro as well as in vivo [1, 10, 12, 13, 16] we tested combinations of alpha- and gamma-IFN and TNF with Vinblastine sulphate (concentration 100 ng/dish, [27]). A synergistic in vitro antiproliferative effect was measured but none of these combinations inhibited colony formation completely (data not shown).

In conclusion, although there were differences in sensitivity between the two renal cell carcinoma xenografts towards alpha- and gamma-IFN and TNF, both lines showed in vitro sensitivity towards the tested BRM's. Most com-

binations of alpha- and gamma-IFN doses of 10 ng/dish and TNF with a dose of 100 ng/dish produced an additive or synergistic effect in the inhibition of in vitro colony formation in soft agar. The combination of alpha-IFN 10 ng/dish and TNF 100 ng/dish had the strongest synergistic effect on colony formation of both tumor lines which resulted in a percentage survival of 0% as compared to the growth control.

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