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Effects of Leukocyte Interferon (*E. coli*) on Human Bone Sarcoma Growth *in Vitro* and in the Nude Mouse*

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Abstract—*Effects of highly purified human leukocyte interferon (rIFN-α2) on colony formation, DNA synthesis and proliferation in nude mice of tumor cells from eight bone sarcomas have been studied. rIFN-α2 produced a dose-dependent inhibition of* [^{3}H]*thymidine incorporation by sarcoma cells. Even at high doses* (10^4 U/ml), *however,* [^{3}H]*thymidine uptake could not be completely blocked by rIFN-α2. In a cloning assay three established sarcoma cell lines and five other sarcoma samples obtained after short-term* in vitro *culture were found to be sensitive to various degrees to rIFN-α2, complete inhibition being seen only at* 10^4 U/ml. *Three sarcomas were sensitive in the nude mouse model. Scheduling experiments revealed that rIFN-α2 produces a delay in tumor growth only when administered either before or shortly after tumor implantation. Therefore rIFN-α2 appears to be most active when tumor size is small and growth not exponential, indicating that rIFN-α2 may play a role in an adjuvant setting. Growth sarcomas strongly suppressed by rIFN-α2 in the cloning assay was markedly inhibited in the nude mouse. One sarcoma which was only moderately sensitive in the cloning assay was resistant in the animal experiment, confirming the predictive value of the clonogenic assay. Although the present findings demonstrate strong antitumor activity of rIFN-α2 against human bone sarcoma cells they should be interpreted with caution mainly because the high rIFN-α2 levels used in the experiments cannot be maintained in patients over a prolonged period.*

INTRODUCTION

INTERFERON (IFN) has demonstrated antiviral [1] immunoregulating [2, 3] and antitumor activity [4]. Inhibition of cell proliferation has been shown on various experimental tumors [2,5-9] and a variety of metastatic tumors in patients [10]. A clinical study of crude leukocyte IFN given as an adjuvant to patients with bone sarcomas has suggested that it may prevent metastatic growth [11].

The recent development in cloning IFN in *Escherichia coli* has made available large amounts of highly purified human leukocyte IFN (rIFN-α2). It was, therefore, of obvious interest to investigate the effects of rIFN-α2 on the growth of human bone sarcoma in a preclinical study. For this purpose, the effects of rIFN-α2 on colony formation, DNA synthesis and proliferation in nude mice of bone sarcoma cells from several patients have been tested.

MATERIALS AND METHODS

Tumor samples used in this study were derived from eight human bone sarcomas established in nude mice. Detailed information regarding source and histology of sarcoma probes is given in Table 1.

Briefly, fresh human sarcoma specimens were cut into small fragments (approximately 1 × 1 × 2 mm) under sterile conditions and implanted subcutaneously into adult nude mice (genetic

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Table 1. Patient and tumor characteristics

Patient	Age/sex	Tumor localization	Tumor type
S	42/M	pelvis	MFH
KN	17/M	femur	OS
T	16/F	tibia	OS
KU	18/M	femur	OS
W	11/F	femur	OS
D	7/F	femur	OS
R	12/M	fibula	EW
C	27/M	scapula	EW

EW = Ewing sarcoma, f = female, m = male, MFH = malignant fibrous histiocytoma, OS = osteosarcoma.

background ICR). The animals were kept under clean conventional conditions. Following 8–12 weeks the sarcomas were serially subpassaged in additional nude mice. After four passages, tumor samples were also processed for *in vitro* cultivation.

Interferon preparation

rIFN-α2 (*E. coli*) with a specific activity of 2×10^8 U/mg protein and purity >90% was kindly provided by Prof. Dr Ch. Weissmann (Zurich).

In vitro *studies*

Human bone sarcomas serially subpassaged into nude mice were explanted under sterile conditions and minced in Iscove's modified Dulbecco's medium (IMDM, KC Biological Inc.). Subsequently, sarcoma tissue was exposed to different enzymes including hyaluronidase, collagenase, dispase and trypsin according to Trechsel *et al.* [12]. Cell suspensions were allowed to settle in plastic bottles (25 cm^3). Adherent cells were further cultivated in IMDM supplemented with 15% fetal calf serum (v/v) and 1% penicillin/streptomycin.

Permanent cell lines were established from three sarcomas (T, KN and S) and were tested for sensitivity against rIFN-α2 after 100 passages. In the other cases (W, D, R, C and KU) rIFN-α2 sensitivity was determined on cells following *in vitro* passage 4 or 5 (referred to as short-term cultures).

Cells growing as monolayers in tissue culture flasks were recovered by adding EDTA–trypsin (GIBCO). Floating free cells were washed twice in culture medium. Cell viability was determined by the trypan blue exclusion method. Single-cell suspensions were subsequently cultured in microtiter wells for determination of [^{3}H]thymidine incorporation and in a semisolid agar cloning system.

For the [^{3}H]thymidine incorporation assay 20,000 sarcoma cells suspended in 150 μl IMDM (supplemented as described) were added to microtiter wells in quadruplicate. After 48 hr at 37°C and 5% CO_2 50 μl of rIFN-α2 were added in three concentrations (10^2, 10^3 and 10^4 U/ml) for 20 hr. Thereafter, 25 μl of 1.5 μCi [^{3}H]thymidine (sp. act. 6.7 Ci/mmol) were added to each well and the plates were incubated for an additional 4 hr. Following two washes with Hank's solution at 37°C, 100 μl 10% trichloroacetic acid (TCA) were added and left for 1 hr at 4°C. After an additional wash with TCA cells were subjected to 2 N NaOH treatment for 1 hr at room temperature. The suspensions were transferred to 2 ml of scintillation liquid (RIA LUMAS) and cpm determined in a Beta-Counter (PACKARD).

In a clonogenic assay rIFN-α2 was added to the cell suspensions and left in continuous contact for the entire culture period. Triplicates of 20,000 cells were suspended in 1 ml of 1.6% methylcellulose containing the same supplemented medium as described above. The suspensions were deposited over a 0.5% agar underlayer. Colony formation was assessed using an inverted microscope; a colony was defined as any cell aggregate >100 micron. Final scoring of rIFN-α2 treated plates and controls was performed between days 5 and 10.

Nude mouse experiments

Each of four bone sarcomas (T, KN, S and KU) was implanted into 8–12 mice, three of which served as controls. Animals were injected subcutaneously with 20,000 U rIFN-α2 dissolved in 0.25 ml phosphate-buffered saline (PBS) daily before and/or after tumor implantation; control animals received only PBS. The size of implants was measured once or twice weekly and tumor volume was calculated according to Machado *et al.* [13].

RESULTS

Effect of rIFN-α2 on DNA synthesis

Suppression of DNA synthesis by rIFN-α2 was tested on three established sarcoma cell lines (T, KN and S). At a low dose, rIFN-α2 depressed [^{3}H]thymidine incorporation to 28–57% (Fig. 1). Higher concentrations did not markedly augment the inhibition of [^{3}H]thymidine uptake and complete suppression was not observed.

Effect of rIFN-α2 on sarcoma colony formation

Five tumor specimens obtained following short-term culture and three others from established cell lines were tested in the cloning assay. The dose-response curves to a continuous exposure of rIFN-α2 are depicted in Fig. 2. All sarcomas were sensitive to rIFN-α2. IFN concentrations of 10^3 and 10^4 U/ml caused an inhibition of colony survival >70% in 3/8 and 5/8 cases respectively.

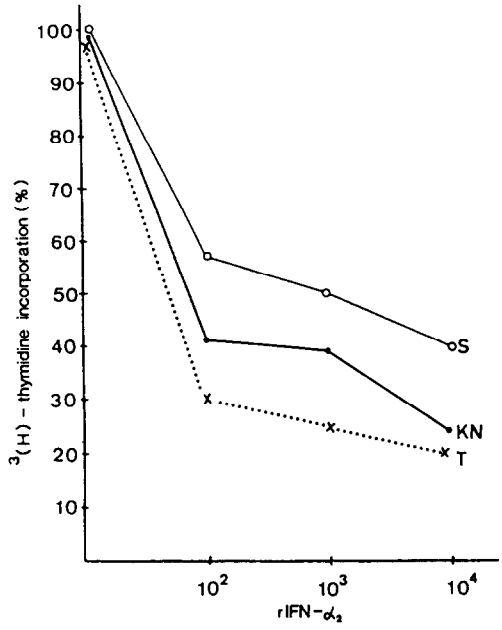

Fig. 1. Effect of rIFN-α2 on DNA synthesis of three established sarcoma cell lines. Each point represents the mean value of four experiments.

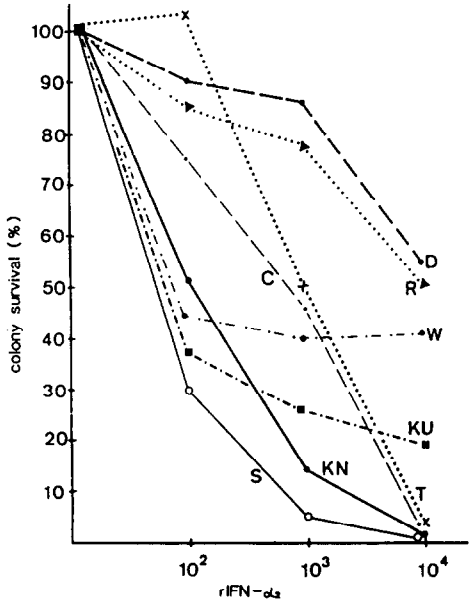

Fig. 2. Effect of rIFN-α2 on colony formation of human bone sarcoma cells. Each point represents the mean value of four assays.

The strongest inhibition of colony formation by rIFN-α2 was observed with the established cell lines (T, KN and S). Response to rIFN-α2 treatment was not asssociated with particular histological features of the sarcoma specimens.

Effect of rIFN-α2 on transplanted sarcoma fragments

The effect of rIFN-α2 was tested in nude mice on the three bone sarcomas (T, KN and S) from which cell lines had been established (Figs 3 and 4). In control animals the three tumors were characterized by an initial slow growth of approximately 14 days followed by a rapid tumor volume increase. Daily applications of rIFN-α2 between days 8 and 23 following implantation caused a delay in tumor growth which became significant after day 21.

An additional sarcoma (KU) which in control animals was characterized by slow growth was not affected by rIFN-α2 (Fig. 3).

The importance of scheduling the application of rIFN-α2 on sarcma growth in nude mice was investigated in the tumor from patient T (Fig. 4). rIFN-α2 administered before and early after tumor transplantation produced the strongest growth delay. Late injections (days 20-29), on the other hand, did not influence tumor volume increase.

DISCUSSION

The results from the present study show that human bone sarcoma cells are sensitive to various degrees to rIFN-α2 as tested in several *in vitro* and *in vivo* systems. rIFN-α2 produced a reduction of cell proliferation which was evidenced by both a decrease in [^{3}H]thymidine incorporation and sarcoma colony formation. This finding supports the view that inhibition of colony formation by rIFN-α2 may be caused by blocking DNA synthesis. Obviously inhibition of DNA synthesis as a consequence of decreased cell multiplication by some other mehanism cannot be excluded from

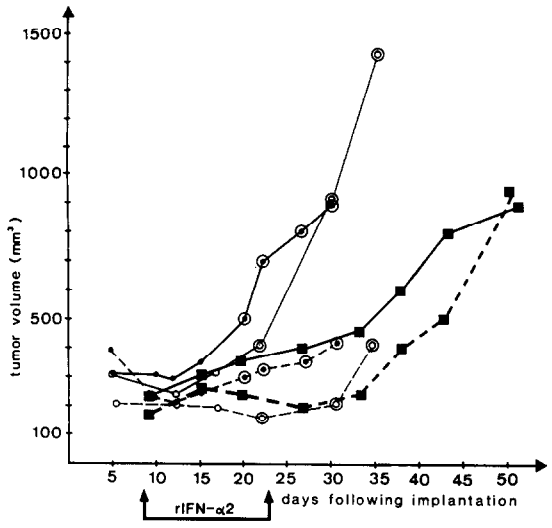

Fig. 3. *Effect of rIFN-α2 on bone sarcoma fragments transplanted into nude mice. The test animals received* 20,000 U rIFN-α2 *daily between days 8 and 23 following implantation. Each point indicates the mean tumor volume of 5-9 test or 3 control animals; encircled symbols represent statistically confirmed* ($P > 0.05$) *differences between tests (continuous line) and controls (broken line).* O——O, *tumor S;* ●——●, *tumor KN;* ■——■, *tumor KU.*

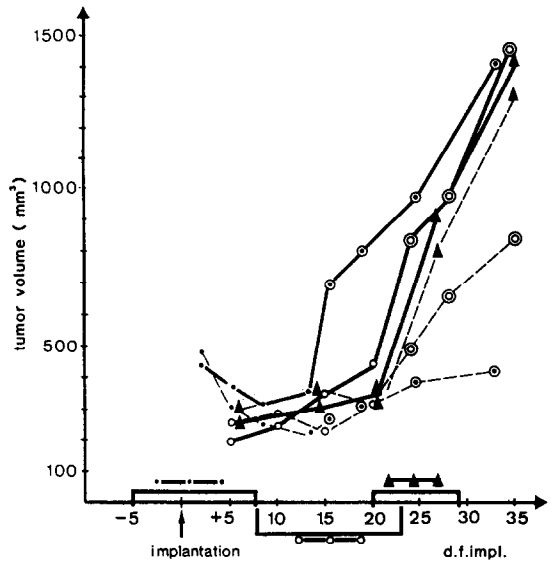

Fig. 4. *Effect of rIFN-α2 scheduling on* in vivo *growth of sarcoma T. Three schedule regimens were tested, each using a daily administration of* 20,000 U rIFN-α2 *subcutaneously:* 5 days *before and continuing* 7 days *after implantation* (●——●——●); *treatment on days* 8-23 (○——○——○); *and rIFN-α2 administration on days* 21-29 *following implantation* (△——△——△). *Control animals are represented by continuous lines, test animals are depicted by broken lines; statistically confirmed differences in tumor volume* ($P < 0.05$) *are indicated by circled symbols.*

our experiments. Other direct effects by rIFN-α2 on sarcoma cells, such as an alteration of the cell surface or cytotoxic damage, may also play a role [14-17].

Results from the *in vitro* experiments suggest that rIFN-α2 may act in the absence of host cells such as natural killer cells [7, 8, 18-21]. Dose-response curves in a cloning assay using continuous exposure reveal that maximal tumor cell kill was only obtained at high rIFN-α2 concentrations (10^4 U/ml). Such high IFN concentrations cannot be achieved over a prolonged period in patients [22].

Sarcoma cells from established cell lines were more sensitive to rIFN-α2 than those from short-term cultures. Strander and Einhorn [23] have also observed a high inhibitory effect of crude leukocyte IFN preparation on established human osteosarcoma cells. The difference in sensitivity observed between sarcoma cells from established lines and from short-term cutures may be related to an increase in IFN receptors following prolonged *in vitro* pasages or other mechanisms.

The *in vivo* relevance of the *in vitro* results was tested in the nude mouse model. We found that three tumors (T, KN and S) which were responsive *in vitro* to rIFN-α2 were also sensitive in the animal model. However, tumor growth was only retarded and required daily rIFN-α2 application of 20,000 U. One sarcoma (KU) which was only moderately sensitive in the cloning assay was insensitive in the nude mouse, emphasizing the relevance of *in vitro* testing.

Scheduling the application of rIFN-α2 in relation to sarcoma inoculation was shown to be of importance. rIFN-α2 produced a delay in tumour growth only when administered either before or shortly after tumor implantation. Treatment started 20 days after sarcoma transplantation was ineffective. These results would militate for an early treatment intervention, when tumor size is small and growth not exponential. Similar observations have been made in a murine osteosarcoma model [24]. Masuda *et al.* [6], on the other hand, studying the effect of rIFN-α2 on one xenografted human osteosarcoma, reported that IFN was active regardless of the treatment scheme. Therefore, bone sarcoma cells may exhibit various patterns of sensitivity against rIFN-α2. The sensitivity differences noted in the cloning assay also suggest that *in vivo* response may vary according to dose, application route and schedule.

This study emphasizes that preclinical data on new drugs should be obtained by different test systems using several tumor specimens of human origin whenever possible. The present findings—although demonstrating clear antitumor activity—should be interpreted cautiously, mainly because high rIFN-α2 doses cannot be given to patients for a prolonged period [22].

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