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Osteosarcoma and interferon

Studies of human xenografts in the nude mouse

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ACTA ORTHOPAEDICA SCANDINAVICA SUPPLEMENTUM NO. 229, VOL. 60. 1989

MUNKSGAARD · COPENHAGEN

**Printed in Sweden
Ortonova
Wallin & Dalholm
1989**

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Introduction

The present work was based on human osteosarcomas transplanted to nude mice. The tumors were maintained as xenografts in mice by serial transplantation. The *aims* were to:

(1) investigate the antitumor effects of human interferon (IFN)- α ; (2) relate IFN- α sensitivity of osteosarcomas to different tumor features; (3) examine the antitumor effects of IFN- γ , given alone and in combination with IFN- α .

Effects of IFN treatment on osteosarcoma xenografts were analyzed in relation to growth, histology, DNA content, and proliferative activity. Characteristics of original osteosarcomas and their xenografts were also analyzed to evaluate the tumor model methodologically. The *aims* were to:

(1) assess the transplantability of human osteosarcomas; (2) identify tumor characteristics related to successful serial transplantation; (3) compare histologic features and DNA content of the xenografts to their original tumors; (4) investigate the relationship between tumor growth rate and proliferative activity of tumor cells; (5) determine the stability of tumor characteristics during long term serial transplantation in nude mice.

Osteosarcoma

Osteosarcoma is a highly malignant neoplasm of bone. Although rare, it is the most common malignant primary bone tumor with an incidence of 2 per 1 million inhabitants in Sweden (Larsson and Lorentzon, 1974). Males are more commonly affected than females and the majority is children and adolescents. Osteosarcoma usually involves the long bones, most commonly about the knee. In treatment series from the 1950's and 60's, less than 20% survived 2 years, despite amputation (Dahlin and Coventry 1967; Marcove et al. 1970). Among those who died, 90% had pulmonary metastases (Jeffree et al. 1975). The poor results were attributed to disseminated disease, undetectable prior to surgery.

Trials on adjuvant chemotherapy in osteosarcoma were begun in the 1970's, aimed at eradicating micrometastases (Cortes et al. 1974; Jaffe et al. 1974; Rosen et al. 1974). Today, the reported 5 year survival ranges between 40 and 70% (Rosen and Nirenberg 1985; Bacci et al. 1986; Simon et al. 1986). The treatment is,

however, associated with severe morbidity, even mortality (Marroum et al. 1977; Rosen et al. 1979). At the Karolinska Hospital, a nonrandomized, consecutive trial of adjuvant IFN treatment in osteosarcoma was initiated in 1971 (Strander 1986). The 7 year survival rate, based on 83 patients treated between 1971 and 1986, was 44% (Bauer 1987). Recent clinical trials for primary osteosarcoma, have contributed to an apparent improved survival through selection of patients with good prognosis (Gill et al. 1988). IFN has not been successful in the treatment of metastatic disease (Strander et al. 1988). Thus, there is no clear clinical evidence proving that IFN can inhibit the growth of osteosarcoma tumors.

Interferon

IFN was discovered in 1957 by Isaacs and Lindenmann. They reported that cells infected by a virus produced a substance which interfered with viral replication, hence the term. It soon became evident that IFNs constitute a complex family of several types and subtypes of cellular proteins. The major types of IFN are designated alpha, beta, and gamma according to antigenic type. Leukocytes produce mainly IFN- α , fibroblasts IFN- β , and T-lymphocytes IFN- γ . By developing recombinant DNA techniques in bacteria, industrial scale production of IFNs is now achieved. The effects of IFN are primarily mediated by binding to cell surface receptors. There is a common receptor for IFN- α and IFN- β while IFN- γ binds to a separate receptor. The IFNs have antiviral activity mediated through cellular metabolic processes. However, IFNs are not only antiviral, but also exert a variety of effects on cells, including inhibition of tumor growth.

Although the antitumor effect of IFN is now well known, the mechanism is as yet poorly understood (Gresser and Tovey 1978; Taylor-Papadimitriou et al. 1985). Experimental evidence suggests that IFNs exert their antitumor activity by growth regulation of cells. The role of host immune mechanism for the antitumor effect is still unclear, but is probably of minor importance (Balkwill and Smyth 1987). The direct action of IFNs on tumor cells has been proven by growth inhibition of neoplastic cells *in vitro* (Paucker et al. 1962; Einhorn and Strander 1978). *In vitro* studies have also

shown that IFN can affect both resting and proliferating cells. Hence, IFN is reported to prolong cell cycle time, inhibit cells from entering the cell cycle, and also block cells in the DNA synthesis phase (Sokawa et al. 1977; Creasey et al. 1980; Balkwill and Taylor-Papadimitriou 1978; Lundblad and Lundgren 1981; Roos et al. 1984). Interestingly, resting cells may be more sensitive to IFN than rapidly multiplying cells (Horoszewicz et al. 1979; van der Bosch and Zirvi 1982).

The antitumor effect of IFN does not only involve growth inhibition but also modulation of cell differentiation. Taylor-Papadimitriou and Rozengurt (1978) concluded that specialized functions of differentiated cells were enhanced by IFN. For example, IFN enhances differentiation of malignant B-lymphocytes *in vitro* (Östlund et al. 1986). These differentiating effects of IFN appears dependent of the degree of cell dedifferentiation; anaplastic cells do not respond (Taylor-Papadimitriou and Rozengurt 1978).

In osteosarcoma, Einhorn and Strander (1978) reported that IFN- α inhibited the growth of human cell lines *in vitro*. There was a wide range in sensitivity to growth inhibition by IFN among the different osteosarcoma cell lines. Transplantable murine osteosarcomas, studied *in vitro* and *in vivo*, can also be growth inhibited by mouse IFN of both the α and γ type (Glasgow et al. 1978). The importance of these findings, regarding the sensitivity of human osteosarcomas to human IFN treatment, is however uncertain. Models of human tumors transplanted to animals might provide more information.

The nude mouse

In 1968, Pantelouris reported that the nude mouse mutant (*nu/nu*) has a congenital absence of the thymus and, consequently, lacks mature T-lymphocytes. This finding suggested that this mouse mutant was deficient in cell mediated immunity. Subsequently, Rygaard and Povlsen (1969) reported that a human colon carcinoma, transplanted subcutaneously to nude mice, grew as a xenograft. Since then, different human neoplasms have been transplanted to nude mice and main-

tained as xenografts in serial passage (Fogh et al. 1982). Benign tumors can grow in one passage, but are not serially transplantable in nude mice.

Within a human tumor transplant, only the neoplastic cells are of human origin, the vascular and connective tissue being derived from the mouse host (Warenius 1980). The neoplastic cells can be divided into 3 groups: proliferating cells (G_1 -S- G_2 /M), resting cells (G_0), and dead or dying cells. Tumor growth is mainly dependent on the relation between the proportion of proliferating cells and the proportion of cells lost due to cell death. Additional factors influencing tumor growth, are extracellular matrix production, oedema, bleeding, and necrosis (Tubiana 1971).

The rationale for employing the nude mouse model in cancer research is based on the assumption that human tumor xenografts retain the characteristics of their original tumors. This is especially important in studies of the effects of antineoplastic agents. Comparative studies of original and transplanted tumors, mainly carcinomas, have shown that the histology, chromosome pattern, and DNA content of the tumor remains unchanged (Fogh and Fogh 1978; Fogh et al. 1982; Rofstad et al. 1982; Neely et al. 1983; Wennerberg et al. 1983; Otto et al. 1984; Clayman et al. 1985; Baisch et al. 1986). The sensitivity of human tumor xenografts to chemotherapy is reported to correlate to experience of clinical treatment of the same tumor entity (Steel et al. 1983; Floersheim et al. 1986).

The nude mouse model is suitable for studies of the effects of human IFNs on human xenografts since IFNs are largely species specific (Uenishi et al. 1983; Heston et al. 1984; Balkwill et al. 1986). Antitumor effects can be regarded as the result of a direct effect of the human IFN on the human tumor cells. Effects of IFN on the immune system are not possible to examine using mice lacking normal T-cell function. Only a few studies have been reported concerning the effect of human IFN on human osteosarcomas transplanted to nude mice (Masuda et al. 1983; Hofmann et al. 1985). In the present study the sensitivity of a series of human osteosarcomas to IFN was related to different tumor features. Furthermore, the nude mouse model was used to investigate tumor and host tissue responses to IFN treatment.

Materials and methods

The study was based on a consecutive series of 32 patients with primary high grade osteosarcoma, treated at the Karolinska Hospital from October 1982 to February 1987. In 7 cases tissue was not procured for transplantation leaving 25 tumors for the present investigation. Tumor tissue was obtained at open biopsy in 4 cases and at definitive surgery in 21. In these 21 cases, adjuvant treatment with IFN- α , at a daily dose of 3×10^6 IU, was begun prior to surgery. No chemo- or radiotherapy was given.

Animals

In all experiments, 6 to 12 weeks old female, BALB/c-*nu/nu* mice (Harlan Sprague-Dawley Inc., Indianapolis, IN, USA) were used. The animals were kept in sterilized cages in a 24°C tempered and isolated laboratory with high efficiency air filtration and 12 hours light and dark cycles. There were 4-10 mice per cage and the mice were fed a sterilized laboratory diet and drinking water. Between 1982 and 1984 no infections occurred. In 1985 the wasting syndrome, common in nude mice (Nomura and Kagiya 1982), was seen in animals more than 4 months old. Therefore, doxycycline (Idocyclin^R, Leo, Sweden) was added to the drinking water at a concentration of 0.02 g/l. With this treatment, the infection rate was reduced from 20% to less than 5%. After moving to a new laboratory in January 1987, there have been no further infections.

Interferon

Both natural and recombinant human interferons were used in this study. The natural IFNs (nIFN) were provided by the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland, and the recombinant IFNs (rIFN) by Boehringer Ingelheim GmbH, West Germany.

Interferon-alpha

The nIFN- α was produced by Sendai virus induction in leukocytes from fresh donor blood, partially purified to a specific activity of 3.3×10^6 IU/mg protein (Cantell et al. 1981a, 1981b). The preparation consisted of more than 20 different IFN- α subtypes, with molecu-

lar weights ranging from 15000 to 28000.

The rIFN- α were derived from *E. coli*, using recombinant DNA technology. The preparation consisted of IFN- $\alpha 2c$ with 165 aminoacid residues. Compared to nIFN- $\alpha 2a$, derived from leukocytes, rIFN- $\alpha 2c$ had an arginine residue, instead of histidine, in position 34 (von Gabain et al. 1986). The specific activity of rIFN- α was 2×10^8 IU/mg protein.

Interferon-gamma

The nIFN- γ was produced from lentil lectin induced leukocytes. The highly purified preparation had a specific activity of 2×10^7 IU/mg protein (Kauppinen et al. 1986). The preparation consisted of three different proteins with molecular weights of 15500, 20000, and 25000, respectively. At least two of these proteins were glycosylated (Kelker et al. 1984).

The rIFN- γ preparation consisted of a 146 aminoacid, non-glycosylated protein (Gray et al. 1982); the specific activity was 2×10^7 IU/mg protein.

Serum concentrations of IFN- γ , of both the natural and recombinant type, were determined after a single subcutaneous injection of 2×10^5 IU to nude mice. At each time point after injection, blood from 3 mice was obtained. After centrifugation, the serum antiviral titres were assayed by a plaque reduction method measuring the cytopathogenic effect of vesicular stomatitis virus in HEp2 cells. The results are presented as the mean of the log values from 2 separate assays of each serum sample. In addition, the serum concentration was assayed using a RIA employing 2 monoclonal IFN- γ antibodies (IRMA, Celltech, England). The IFN titres were expressed in IU/ml serum. The laboratory standards was calibrated to NIH IFN- γ reference standard Gg 23-901-530.

Tumor transplantation

The tumor sample was placed in sterile saline solution, 4° C. Within 3 hours, the tumor tissue was cut into pieces, 1-2 mm in diameter, and transplanted to nude mice. 4 mice received 2 tumor pieces each, placed subcutaneously over the right and left flank. The remaining tumor tissue was prepared for histologic examination and flow DNA cytometry.

Transplantation of tumor tissue between mice was performed when the xenografts reached a diameter of approximately 15 mm. Immediately after death by cervical dislocation, tumor tissue was removed and inoculated in new mice. There was no intended selection of slow or fast growing tumors. Peripheral tumor tissue was chosen to avoid central necrotic areas.

Interferon experiments

In each experiment, 20 to 60 mice were inoculated with 1 piece of tumor tissue. Only mice exhibiting measurable tumor growth within 3-5 weeks after transplantation were used in the subsequent experiments. The tumor bearing mice were divided into 1 control group, receiving human serum albumin and 1 or more IFN treatment groups. The mice were allocated to the different groups so that the mean tumor volume of each group at the start of treatment was approximately equal. The IFN treatment was given as subcutaneous injections over the neck of the mice, *i.e.*, distant from the tumor. The IFN preparations were diluted so that the administered volume was 0.1-0.2 ml. The given dose was expressed in international antiviral units (IU). Treatment was discontinued when the size of the control xenografts was 1-5 cm³. After completion of each experiment, tumor tissue was prepared for histologic examination and DNA analysis.

Growth rate

Tumor size was assessed using a slide caliper at regular intervals after transplantation. The tumor volume was calculated as: $\pi \times a \times b \times c / 6$, where *a*, *b*, and *c* represent the diameters in 3 planes. This assessment of volume correlated strongly to the wet weight of the tumors (Figure 1). Growth rate was expressed by the volume doubling time, calculated as: $\log 2 \times (t_1 - t_0) / (\log v_1 - \log v_0)$ where *v*₀ was the tumor volume at day *t*₀ when a measurable tumor was first observed, and *v*₁ was the volume on day *t*₁ when the mice were killed. In experiments with IFN, growth rate was expressed as the Growth Index, derived by dividing the tumor volume at the end of IFN treatment with its volume at the start of treatment.

Histology

The tumors were fixed by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7 for at least 24 hours at room temperature (Karnovsky 1965). Some specimens were subsequently demineralized in 22% formic acid and 10%

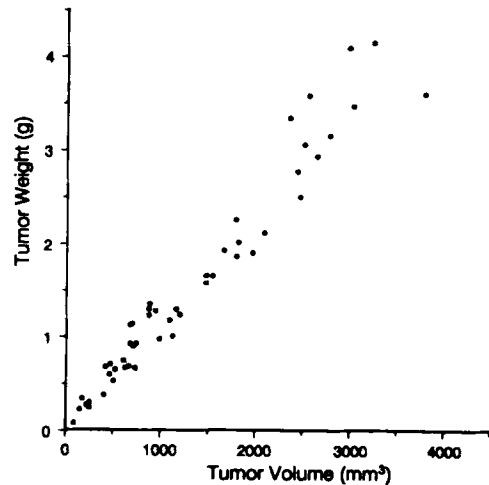


Figure 1. Correlation between tumor volume and tumor wet weight of 50 tumors from the xenografts T8, T11, and T12. Regression analysis: $Y = 0.06 + 1.14X$; $r = 0.97$; $p < 0.001$.

sodium citrate in water pH 1.9 for 1-3 days. The specimens were then dehydrated by serial washing with ethanol, graded 70 to 95%, and embedded in glycol-methacrylate (JB-4, Polysciences Inc., England). Approximately 1.5 μ m thick sections were cut with a Sorvall JB 4A microtome from 1-3 blocks representing central and peripheral parts of the tumor, and stained with hematoxylin and eosin.

Original tumors, and their respective xenografts, were classified according to histologic subtype (osteoc, chondro-, and fibroblastic; Dahlin 1975), and Grade (I-IV; Broders et al. 1939). Furthermore, the Mitotic Index was calculated according to the number of mitotic figures per 20 high power fields of vision, corresponding to a total of 1500-2000 cells. The fields of vision were systematically random sampled within the most cellular areas of the tumor (Price 1961). The histologic assessment was performed, without access to the experimental protocols, by Finn P. Reinholdt, M.D., Department of Pathology, Huddinge Hospital.

Flow DNA analysis

The tumor specimens were squeezed through nylon gauze with isotonic Tris-EDTA buffer pH 7.5. After centrifugation, the cells were fixed in ice cold 96% ethanol. The fixed cells were washed in Tris-EDTA buffer with 1 mg/ml RNA-ase. Suspensions of single cell nuclei were obtained by pepsin treatment and stained using 2×10^{-5} Methidium bromide in Tris-EDTA buffer with a molarity of 395 mOsm.

Table 1. Channel number corresponding to the DNA content of G₀/G₁ cells in flow DNA cytometric analyses of control human lymphocytes and tissue from BALB/c-*nu/nu* mice. Values are the mean of analysis of 3 specimens of each tissue type

Tissue	Channel number	Coefficient of variation (%)
Human lymphocytes	69	3.5
Mice spleen	69	6.3
Mice small intestine	70	3.0
Mice muscle	70	3.8

The DNA content of the cell nuclei was determined in a flow cytometer ICP 11 (Ortho Instruments, U.S.A) equipped with a Xenon lamp (Osram XBO 75 W). The excitation and emission wavelengths were 455-490 nm and 590-630 nm, respectively. Output was sorted with a 256 multichannel analyzer. Human lymphocytes, isolated in a Ficoll gradient and prepared at the same time as the tumor material, were used as external staining controls. The amplitude of the light pulses from the lymphocytes was adjusted to channel number 70. The coefficient of variation was less than 3%. In analysis of tumor samples, the channel of the control lymphocytes was sometimes adjusted to 40 or 28, because of high amplitudes of tumor cell nuclei. For further details see Tribukait 1987.

DNA content

The channel number of the control lymphocytes was denoted DNA Index (DI) 1.0, representing the normal diploid DNA content of human cells. In the analysis of nondiploid original tumors, a diploid peak was also present, considered to represent nonneoplastic cells, always present in the tumor sample. In the analysis of the osteosarcoma xenografts, a diploid peak was also consistently found, representing admixed normal mouse cells or diploid human tumor cells. DNA analysis of different types of normal tissue from BALB/c nude mice revealed that the G₀/G₁ peak of mouse cells was present in the same channel as human lymphocytes (Table 1). Hence, in contrast to previous studies (Wennerberg 1984; Baisch et al. 1986), it was not possible to distinguish between normal host mouse cells and diploid human cells.

The DNA values of the analyzed cells from original tumors and their xenografts were expressed in relation to the DNA value of the control lymphocytes. Tumors with a unimodal peak within 10% of the diploid standard were classified as diploid (DI 0.9-1.1). Tumors were classified as aneuploid in the presence of non-diploid peaks. Furthermore, tumors with a peak at DI

1.9-2.1 containing >15% of the analyzed cells, and a corresponding (G₂/M) peak in the octaploid region, were also classified as aneuploid (Tribukait 1984).

The proportion of cells in the various phases of the cell cycle was determined according to the method of Baisch et al. (1975), after correction for background disturbance. Cell cycle analysis was based on the aneuploid cell population.

Slide DNA analysis

Tissue sections were prepared according to Kreicbergs and Zetterberg (1980). Fresh tumor tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 4 µm were deparaffinized in xylol for 20 min, and refixed in 10% formalin before Feulgen staining. Additional sections were stained in hematoxylin-eosin for histologic identification of areas for DNA measurement.

Staining of cell nuclei was performed using a modified Feulgen technique, i.e., acid hydrolysis in 5 M HCl, 1 hour, 22° C before staining (De-Cosse and Aiello 1966; Eneroth and Zetterberg 1974).

The DNA measurements were taken in an Image Analyzer (Ahrens system, Hamburg, W. Germany), attached to a photomicroscope, with an objective lens x 40 (Nikon, Japan) attached to a Video-CCD array camera (JVC 500). Tissue sections were scanned at a wavelength of 546 nm and optical density was measured and integrated. Nude mouse myocytes, fixed and prepared simultaneously with the tumor tissue, were used as staining controls. 50 control cells and 100 tumor cells were analyzed in each tissue section.

DNA content

The median (50th percentile) DNA content of each control cell population was given the arbitrary value DNA Index (DI) 1.0, denoting the diploid DNA content. The proportion of nondiploid cells in each tumor cell population was determined by calculating the percentage of cells with DNA values exceeding DI 1.25 (Erhardt et al. 1984, Bauer et al. 1986a).

Statistics

The following statistical methods were used: the 2 tailed Student's *t*-test, the Wilcoxon's 2 tailed rank sum test of nonparametric data, the chi-square test and a linear regression analysis. The levels of statistical significance were: **p*<0.05, ***p*<0.01, ****p*<0.001 respectively. *p*>0.05 was regarded as not significant.

Transplantation to nude mice

The establishment rate of different human malignant tumors as serial transplants in nude mice varies considerably. In this study the take rate of human osteosarcomas, transplanted to nude mice, was assessed to identify tumor characteristics related to successful serial transplantation. Histology and DNA content of osteosarcoma xenografts were also compared to their original tumors (Bauer et al. 1986b).

Original tumors

The series was based on 25 osteosarcomas that were transplanted to nude mice. Histologically, 11 were classified as osteoblastic, 9 as chondroblastic and 5 as fibroblastic. All of the lesions were of high grade, 13 Grade III and 12 Grade IV. Flow DNA analysis disclosed that 24 were aneuploid and only 1 was diploid. Hence, the original tumors were characterized histologically by high grade and cytochemically by an abnormal DNA content.

Take rate

Tumor growth, of at least 1 out of 8 transplanted tumor pieces, was evident within 3 to 8 weeks after transplantation of 16 osteosarcomas. However, in 2 cases tumor growth was very slow and ceased in the second passage. There was no measurable growth, during an observation period of at least 4 months, after transplantation of the remaining 9 osteosarcomas. Hence, 14 out of 25 transplanted osteosarcomas grew in nude mice, *i.e.*, the take rate was 0.6. These 14 osteosarcomas were designated T1 to T14. The remaining 11, including the 2 tumors that only grew in the first passage, were classified as nontransplantable.

The original tumors of the transplantable group had a similar histologic subtype distribution to the non-transplantable osteosarcoma group. However, there was a predominance of Grade IV lesions, 9 out of 14, in the transplantable group, as compared to 3 out of 11 in the non-transplantable (chi-square 3.6, $p=0.06$). There was no apparent relation between ploidy level and transplantability. The transplantable osteosarcomas had a mean percentage of S-phase cells of 22 (SD 8.3) as compared to 15 (SD 3.5) in the non-transplantable ($p<0.05$) (Table 2). This comparison was based on only 19 tumors, since cell cycle analysis was not con-

sidered feasible in 6 due to high background disturbance and/or low proportion of aneuploid cells.

In summary, the take-rate of transplanted high grade osteosarcomas to nude mice was 0.6. The transplantable tumors appeared to have higher proliferative activity than the nontransplantable. The following studies were based on the 14 osteosarcomas, T1-T14, established as xenografts in nude mice.

Transplanted tumors

Growth rate

The osteosarcomas grew exponentially in nude mice (Figure 2). The growth rate of the 14 osteosarcomas in the first passage, expressed as the volume doubling time, ranged from 6 to 23 days.

Histology

The osteosarcomas grew subcutaneously as lobulated tumors within a pseudocapsule. The periphery of the tumors was highly cellular and vascular, whereas in the central areas more extracellular matrix and, occasionally, necrosis were seen. The tumors did not

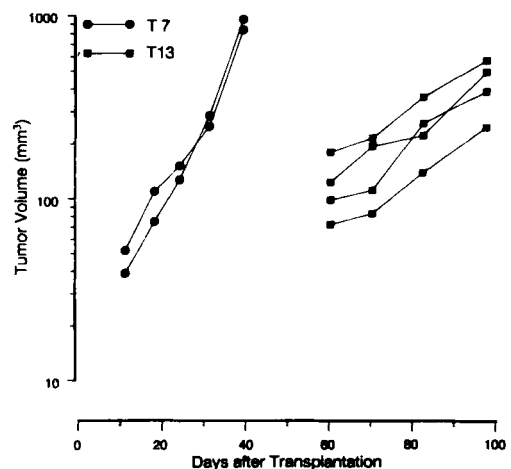


Figure 2. Growth curves of tumors from osteosarcomas T7 and T13 in first passage in nude mice. Each line represents one tumor.

Table 2. Histologic and flow DNA cytometric data from the original tumor specimens of 25 osteosarcomas

Tumor	Type ^a	Grade	Cells in S-phase (%) ^b	DNA Index	Number of passages in mice
Transplantable					
T1	O	IV	15	1.0+2.0	22
T2	O	III	11	1.8	21
T3	F	III	15	1.9	9
T4	O	IV	44	1.3	10
T5	O	IV	21	1.5	13
T6	C	IV	22	1.4+2.8	6
T7	C	III	23	1.1	13
T8	C	III	19	2.4	18
T9	C	III	NA	0.9+1.8	7
T10	C	IV	21	1.6	4
T11	F	IV	28	1.7	14
T12	O	IV	23	2.8	5
T13	O	IV	NA	1.6	2
T14	C	IV	20	1.3	2
			Mean SD	22 8.3	
Non-transplantable					
T15	F	IV	18	1.0+2.0	-
T16	O	III	NA	1.6	-
T17	O	III	17	1.7	-
T18	F	IV	17	1.4	-
T19	O	III	18	2.1	-
T20	C	III	12	1.8	1
T21	O	IV	NA	2.2	-
T22	C	III	14	1.1+2.3	-
T23	C	III	9	1.9	-
T24	F	III	NA	1.0	-
T25	O	III	NA	2.3	1
			Mean SD	15 3.5	

^a O osteoblastic, C chondroblastic, F fibroblastic.

^b NA not possible to assess due to low proportion of aneuploid cells and/or high background disturbance. The transplantable tumors had higher proportion of S-phase cells as compared to the non-transplantable ($p < 0.05$; Wilcoxon's rank sum test).

invade adjacent structures and metastases were not observed.

Comparison of original tumors with their respective xenografts disclosed identical histologic subtype and grade. There were 6 osteoblastic, 6 chondroblastic, and 2 fibroblastic tumors; 5 were Grade III and 9 Grade IV.

DNA analysis

Flow DNA cytometry showed that the ploidy characteristics of the original tumors were retained after transplantation. Hence, the ploidy level was the same, as compared to their original tumor, in 12 out of 14 xenografts. There were, however, 2 exceptions. The original lesion T9 exhibited 2 aneuploid cell populations, at DI 0.9 and 1.8 whereas in the xenografts only 1 of these, at DI 1.8, was seen (Figure 3). The other exception was T7; the original tumor was aneuploid (DI 1.1), but the xenograft diploid.

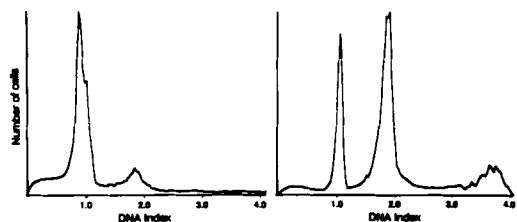


Figure 3. Representative DNA histograms of osteosarcoma T9. Original lesion (left) and tumor from first passage in nude mice (right).

The mean proportion of S-phase cells of the osteosarcomas in the first passage was 20% (SD 5.6), compared to 22% (SD 8.3) in the original tumors. However, regression analysis showed no significant correlation for individual osteosarcomas ($n=12$; $r=0.41$; $p>0.05$). For comparison, regression analysis between the percentage of S-phase cells of osteosarcomas in the first and second passage revealed a correlation ($n=14$; $r=0.69$; $p<0.01$).

This study showed that the osteosarcomas, transplanted to nude mice, were heterogeneous with respect to the analyzed tumor characteristics: growth rate, histologic subtype, DNA content, and proliferative activity. However, the xenografts, in the first passage in nude mice, essentially retained the characteristics of their original tumor.

Comments

The take rate after transplantation of human malignant tumors to nude mice varies for different tumors. In general, the rate increases with increasing degree of malignancy (Fogh et al. 1982); histologically low grade lesions can seldom be established in multiple passages (Fogh et al. 1980; Clayman et al. 1985). The take rate may therefore be related to the proportion of high grade lesions within the tumor entity. Transplantability to nude mice has been reported to be a negative prognostic factor in renal cell carcinoma (Otto et al. 1984; Clayman et al. 1985) and osteosarcoma (Sasaki et al. 1986).

In this study of human osteosarcomas transplanted to nude mice, the take rate was 0.6. For comparison, Fogh et al. (1982) reported an over all take rate of 0.3 after transplanting 381 tumors of 14 different entities to nude mice. The high rate reported here may be due to the fact that all the osteosarcomas were of high grade malignancy; all were Grade III or IV lesions and all except 1 had an abnormal DNA content. In a similar study of osteosarcoma, Sasaki et al. (1986) reported a take rate of 0.3. This low rate, as compared to the present, was probably due to differences in transplanta-

tion technique. In the present study, 8 pieces of tumor tissue was transplanted to 4 mice in each passage whereas Sasaki and co-workers only transplanted 3 samples.

The 14 transplantable osteosarcomas had higher proportion of S-phase cells as compared to the non-transplantable. As reported in other tumor entities (Fogh et al. 1982; Clayman et al. 1985), it appeared to be the most anaplastic lesions that were transplantable. Thus, the 14 osteosarcomas established in multiple passages can be considered to represent the most malignant tumors of the series. This selection, inherent in the nude mouse model, should be taken into account in the further studies of these tumors.

The 14 osteosarcomas exhibited differences in histologic differentiation, ploidy level, and growth rate, even though all were highly anaplastic. The characteristics of the xenografts were the same as those of their respective original tumors. Hence, the xenografts exhibited unchanged histologic appearance and DNA content. However, the proportion of S-phase cells, representing an estimate of the proliferative activity, did not correlate between original and transplanted tumors. This discrepancy may be due to altered site of tumor growth, but was more likely due to specimen differences. In the original tumors, the proportion of aneuploid cells was generally lower, compared to the xenografts, and there was also higher background disturbance due to cell debris. Together these two features made the assessment of cell cycle proportions in original tumors uncertain. The xenografts, on the other hand, provided better cell material for analysis with a low proportion of diploid cells and low background disturbance. The reliability of cell cycle analysis, based on the xenografts, was evidenced by the correlation between the S-phase cell assessments in the first and second passage.

This study shows that human osteosarcomas, transplanted to nude mice, retain the basic tumor characteristics of the original tumor. In the following section, the tumor features were analyzed during serial transplantation.

Serial transplantation in nude mice

Previous studies of human xenografts, mainly carcinomas, have shown essentially unchanged tumor features during serial passage in nude mice (Fogh et al. 1982; Neely et al. 1983; Wennerberg et al. 1983; Otto et al. 1984; Clayman et al. 1985). However, DNA analysis by flow cytometry have revealed that changes in ploidy level can occur (Rofstad et al. 1982; Baisch et al. 1986).

In this study, characteristics of human osteosarcomas were assessed during serial transplantation in nude mice (Bauer et al. 1986b). In addition to investigating the relationship between tumor growth and proliferative activity, the aim was to determine the stability of DNA content and histologic features in osteosarcoma xenografts.

Growth and proliferation

Analysis of the relationship between tumor growth rate and cell proliferation was based on tumors from the first two passages in mice. The mean growth rate, assessed as the tumor volume doubling time, ranged from 6 to 21 days. The proportion of cells in S-phase of

the cell cycle ranged from 9 to 30%, and the Mitotic Index from 5 to 32. There was no apparent relationship between the growth rate and the two parameters of cell proliferation employed (Figure 4). However, corresponding analysis of S-phase cell percentages and Mitotic Indices showed that these 2 different methods of assessing proliferative activity correlated.

These findings show that the tumor growth is not directly related to cell proliferative activity. Furthermore, the correlation between the proportion of S-phase cells and Mitotic Index, indicate that they represent valid, although rough, estimates of cell proliferation.

Number of passages

Among the 14 transplantable osteosarcomas, 2 are still growing in nude mice, whereas the transplantation of 4 has been intentionally discontinued. Serial transplantation of the remaining 8 osteosarcomas ceased, partly due to host infection.

3 osteosarcomas (T1, T2, and T8) were serially transplanted from 18 to 22 passages, for more than 3

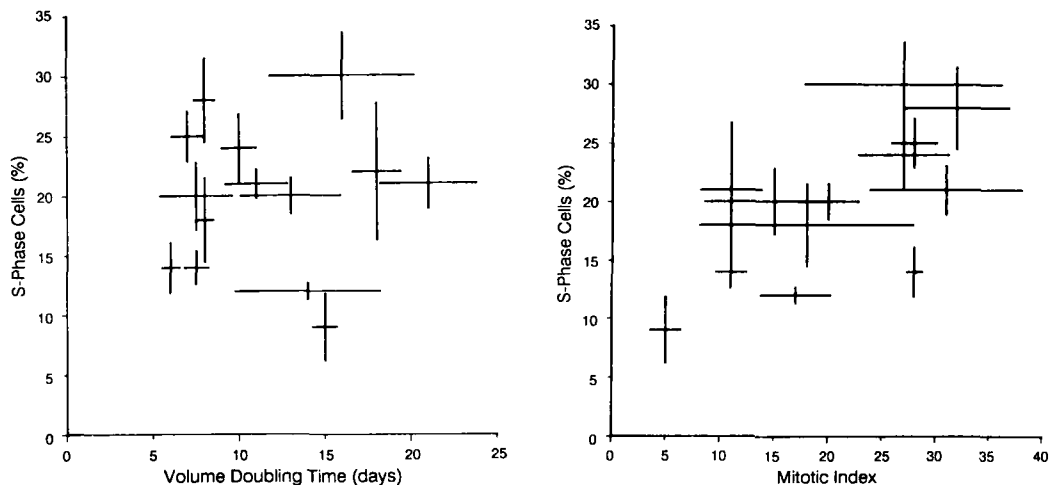


Figure 4. Relation between proportion of S-phase cells and volume doubling time (left). Regression analysis: $n=14$; $r=0.023$; $p>0.05$. Relation between proportion of S-phase cells and Mitotic Index (right): $n=13$; $r=0.675$; $p<0.01$. The intersections represent the means and the lines 2SD.

Table 3. Growth rate and flow cytometric data of 7 osteosarcomas with unchanged DNA content during serial transplantation

Tumor	Number of passages	Volume doubling time (days) ^a	DNA Index ^b	Cells in S-phase (%) ^a
T2	21	8.2 2.1	1.8-1.9	14 3.0
T3	9	15.1 3.2	1.9-2.0	7 2.9
T4	10	8.7 2.9	1.3-1.4	25 4.5
T5	13	10.3 3.1	1.4-1.5	20 3.2
T8	18	7.8 0.9	2.3-2.5	21 4.8
T9	10	8.9 1.6	1.7-1.8	20 5.5
T10	4	19.4 3.1	1.6-1.7	16 4.5

^a Mean SD.^b Range.

years, whereas 9 have been passaged from 4 to 14 times during 15 to 30 months (Table 3 and 4). Osteosarcomas T13 and T14 are presently in the third passage in nude mice.

Take rate

Assessment of take rate was based on the transplantation of at least 8 pieces of tumor tissue in every passage, *i.e.*, 2 transplants were inoculated in each of 4 mice. The take rate varied among the 14 osteosarcomas, but was fairly constant from passage to passage. The highest take rate, 0.8-0.9, was noted after transplantation of T1, T7, T8, T11, and T12. Osteosarcomas T3 and T6 had the lowest take rate, approximately 0.5. Osteosarcomas exhibiting the lowest take rates had low growth rates.

Tumor features

In 7 of the 12 osteosarcomas studied in more than 2 passages, the assessed tumor characteristics remained unchanged throughout the observation period (Table 3). Hence, the histologic appearance and DNA content were found to be stable features of these osteosarcomas. For example, in the first passage of T8, histologic examination disclosed a Grade III chondroblastic tumor, and DNA analysis an aneuploid cell population at DI 2.4. During 3 years of transplantation in nude mice, corresponding to 18 passages, the histologic appearance was unchanged. Furthermore, the growth rate, ploidy level, and proportion of S-phase cells exhibited only small variations from passage to passage (Figure 5).

However, major changes in tumor characteristics were seen in 5 other osteosarcomas (Table 4). In 4 of

Table 4. Growth rate and flow DNA cytometric data of 5 osteosarcomas exhibiting changes in DNA content during serial transplantation

Tumor	Passage numbers	Volume doubling time (days) ^a	DNA Index	Cells in S-phase (%) ^a
T1	1-16	5.8 2.2	1.0+2.0	22 3.3
	17-22	5.3 1.6	1.8	27 3.3
T6	1-2	19+13	1.4+2.8	30+30
	3-6	8.5 2.5	2.8	22 5.5
T7	1	6.4	1.0	15
	2-3	5.7+7.2	1.0/2.0	12+16
	4-13	4.7 1.1	1.0	16 3.1
T11	1-6	7.3 1.2	1.7	23 2.7
	7-11	7.0 0.7	1.7+3.2	25 3.7
	12-14	6.0 0.2	2.7	29 5.5
T12	1-4	12.2 3.7	2.8	18 3.7
	5-6	8.9+7.1	2.7	31 4.7

^a Mean SD.

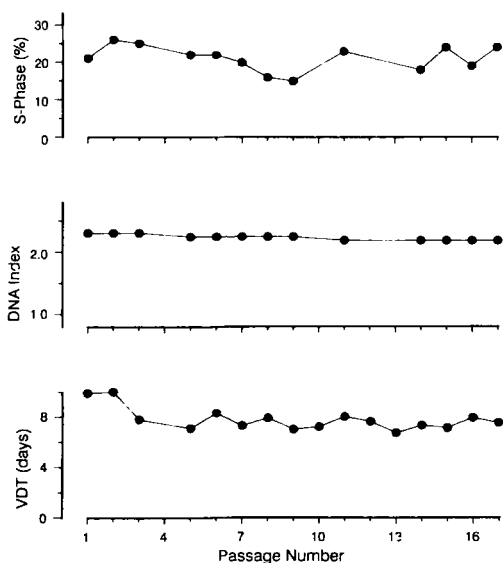


Figure 5. Growth rate, DNA Index, and proportion of S-phase cells of osteosarcoma T8 during serial transplantation in nude mice for 3 years. Each point represent the mean of 2-7 tumors in each passage.

these (T1, T6, T7, and T11), the DNA content of the tumors changed, and in T12 the proportion of S-phase cells increased. Concomitantly, the growth rate of these 5 osteosarcomas also increased, but, histologically, neither type of tissue differentiation nor malignancy grade changed.

The osteosarcoma T1 was originally an aneuploid tumor (DI 2.0). However, in the first 16 passages, the DI 1.0 peak predominated, with only 15 to 25% aneuploid cells. During passages 17 to 22, a gradual reduction of the DNA content was seen, from DI 2.0 to DI 1.8. Concomitantly, the size of the aneuploid cell population increased, to approximately 80% of the analyzed cells.

Changes in the DNA content of osteosarcoma T7 exhibited a different pattern (Figure 6), partly due to unintentional selection of tumors for transplantation. The original lesion was aneuploid, DI 1.1, whereas the tumors in the first passage were diploid. In the second passage, 1 of the 2 analyzed tumors exhibited an aneuploid cell population (DI 2.0) whereas the other remained diploid. Tissue from both these tumors were transplanted to the third passage, resulting in 2 diploid and 2 aneuploid tumors. The tumor used for transplantation to the fourth passage was found to be diploid. In

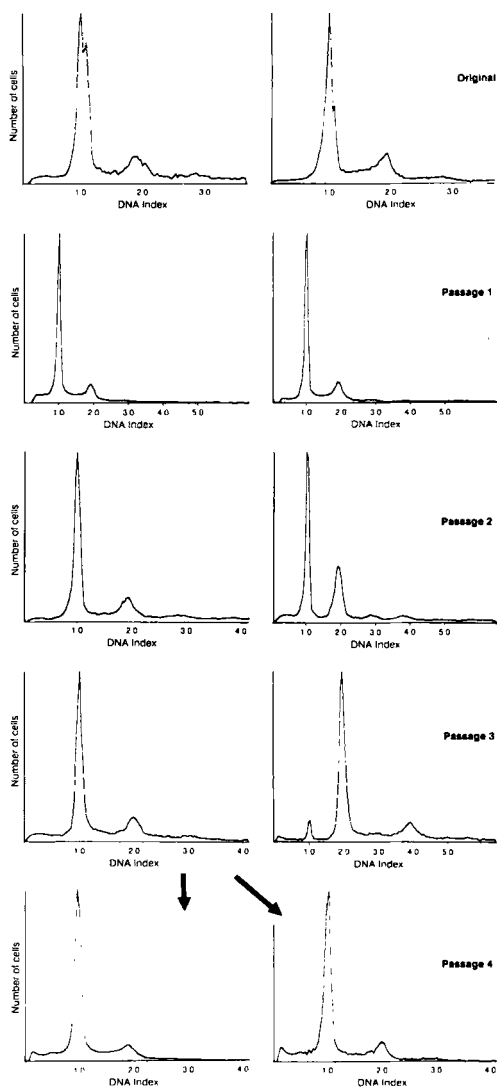


Figure 6. Representative DNA histograms of osteosarcoma T7 during serial transplantation in nude mice. Arrows represent the unintentional selection of a diploid tumor for transplantation to the next passage. Note the different scaling for the DNA Index.

this and the following 8 passages, none of the analyzed tumors exhibited an aneuploid cell population, i.e., all tumors were diploid. The growth rate of this xenograft gradually increased.

In 2 other tumors exhibiting changes in DNA content (T6 and T11), an increase in ploidy level was observed. Osteosarcoma T6 had 2 aneuploid cell populations at DI 1.4 and 2.8 in the original tumor and in tumors of the first 2 passages in mice. In the third pas-

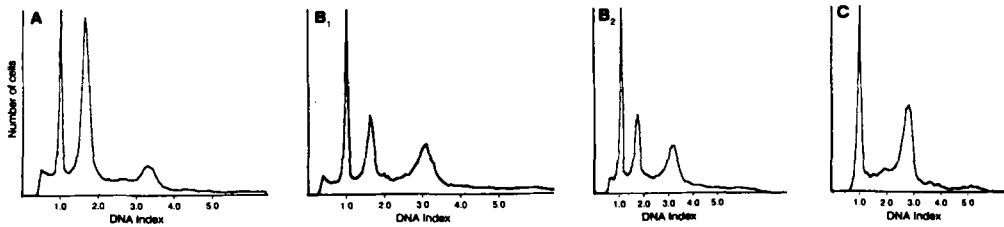


Figure 7. Representative DNA histograms of osteosarcoma T11 during serial transplantation in nude mice. Note the DNA peak 1.7 in passage 5 (A); peaks 1.7 and 3.2 in passage 7 (B1 and B2; different scaling for the DNA Index); and peak 2.7 in passage 12 (C).

sage, the peak at DI 2.8 dramatically increased in size; the DI 1.4 peak subsequently disappeared in the following passages. The tumor growth rate doubled at the same time as the change in ploidy level. Regarding T11, only 1 peak, at DI 1.7, was seen in the first 6 passages. In the seventh passage, 2 peaks were encountered, a new one at DI 3.2, besides the original peak. In the following passages, the DI 1.7 peak gradually disappeared, and in passage 12 was completely replaced by the peak with the higher DNA value (Figure 7). Subsequently, the DNA content of this cell population gradually decreased, from DI 3.2 to DI 2.7. Simultaneously, the tumor growth rate increased, from a volume doubling time of 7.3 to 6.0 days. Finally, in T12, the DNA content did not change, but the proportion of S-phase cells increased, from 18%, in the first to fourth passage, to 31% in the fifth and sixth passage. This related to increased tumor growth, *i.e.*, a reduction in volume doubling time from 12 to 8 days.

This study of osteosarcomas in serial passage in nude mice, showed that histologic appearance remained unchanged. Furthermore, ploidy level was stable in 8 osteosarcomas. In the remaining 4, the changes in DNA content appeared to be the result of a selection of one of two cell populations, coinciding with increased growth rate.

Comments

The nude mouse model permits repeated studies of the same tumor by serial transplantation over an extended time period. The risk of losing a human tumor xenograft is dependant on take rate and the number of transplanted tumor specimens in each passage. In the present study, the lowest take rate was 0.5 and 8 tumor pieces were transplanted in each passage. In these circumstances, the risk of inadvertently losing a xenograft during the first 10 passages is only 0.04. If, for example, only 3 tumor pieces are transplanted in each passage, the risk would be 0.74. Therefore, the number

of transplanted tumor pieces in the current study appears sufficient. There have been reports of serial transplantation of a human melanoma, a colon carcinoma, and renal cell carcinomas in more than 50 passages in nude mice (Vindelöv *et al.* 1982; Spang-Thomsen and Vindelöv 1984; Baisch *et al.* 1986). In this study, 3 osteosarcomas grew in 18 to 22 passages for more than 3 years, suggesting that some xenografts can grow indefinitely.

The growth rate of osteosarcomas in nude mice was similar to other human xenografts, such as melanomas and renal cell carcinomas (Rofstad *et al.* 1982; Baisch *et al.* 1986). The volume doubling times, in the present and previous studies, were less than 30 days. The growth rate of osteosarcomas in nude mice was faster than for osteosarcoma metastases in man. Charbit *et al.* (1971) reported a mean volume doubling time of 40 (range 8 - 320) days for sarcoma lung metastases. This difference in growth rates may be attributed to the selection of fast growing tumors inherent to the nude mouse model.

The correlation between the two different methods of estimating proliferative activity implies that they provide conclusive information. However, both methods have methodological errors. In assessing the S-phase percentage from DNA histograms, errors are caused by high background disturbance, admixture of normal cells, and problems in delineating the different phases of the cell cycle. Xenografts provided better tumor tissue material in this respect, with lower background disturbance and fewer diploid cells. Tissue fixation and preparation can cause major errors in calculating the Mitotic Index. In the current study, tumor specimens were fixed in neutral para-formaldehyde-glutaraldehyde (Karnovsky 1965) and embedded in glycolmethacrylate (Weibel and Knight 1964). These methods are associated with less artefacts compared to conventional techniques, *i.e.*, formalin fixation and paraffin embedding (Graem and Helweg-Larsen 1979). Nevertheless, the Mitotic Index can only provide a rough estimate of the proliferative activity

because of other methodological problems, such as representativeness of the tissue sample and differences in cell density.

Neither the proportion of S-phase cells nor Mitotic Index of the osteosarcomas correlated with tumor volume doubling time. This finding is probably due to the fact that tumor growth is dependent of cell loss, extracellular matrix formation, and other factors, besides cell proliferative activity (Tubiana 1971). Variations in cell loss fraction have been implicated as the most important factor for differences in growth rates of xenografts (Rofstad et al. 1982). Nevertheless, the growth rate may be related to the degree of tumor differentiation. In the present study, relatively well differentiated tumors, with abundant osteoid formation, had lower growth rates.

Osteosarcomas exhibited stable tumor features during serial passage in nude mice, as assessed histologically. Hence, osteosarcomas displayed the same type of tissue differentiation, characteristic for osteosarcomas of different subtypes, from passage to passage. This finding suggests that the histologic subtype, which is one expression of neoplasia, is a highly stable tumor feature. In fact, this consistency in histologic appearance remained, even when changes in DNA content were encountered.

The ploidy level of osteosarcomas also remained stable during serial transplantation in mice. In 8 of the 12 osteosarcomas studied for more than 1 year, changes in DNA content were not observed. Furthermore, in 2 (T1 and T6) of the 4 osteosarcomas with ploidy alterations, the changes appeared to be the result of selection of a preexisting, but minor cell population. The finding that osteosarcomas retain their individual aneuploid feature during serial transplantation, agrees with similar studies of renal cell carcinomas and malignant melanomas (Rofstad et al. 1982; Baisch et al. 1986). Comparative DNA analysis of primary and recurrent human malignancies, have also demonstrated unchanged DNA content during progression of

disease (Auer et al. 1980; Friedlander et al. 1984), including osteosarcoma (Bauer et al. 1988). The combined findings from these clinical and experimental studies show that aneuploidy is a relatively stable neoplastic expression. However, significant changes in DNA content may occur.

In this study, the changes in DNA content observed in 4 osteosarcomas, can be regarded as an illustration of the development of aneuploidy in malignant tumors. Although the alterations in DNA content were quite different in the 4 tumors, the common mechanism for the appearance of a new and dominant cell population seems to be polyploidization, followed by a selection of the cell population with the higher DNA content (Mitelman 1972; Nielsén 1976; Vindelöv et al. 1982; Baisch et al. 1986).

In several malignant tumor entities, including sarcomas, the majority of aneuploid lesions has a DNA content in the triploid to tetraploid range (Kreicbergs et al. 1987; Tribukait 1987; Bauer et al. 1988). One possible explanation for this finding is polyploidization of near-diploid lesions, subsequently followed by DNA losses (Tribukait 1984). The findings of the present study supports this hypothesis. For example, during serial transplantation of T1, the tetraploid cell population became dominant, and simultaneously the DNA content decreased to DI 1.8, *i.e.*, an aneuploid cell population developed from a diploid-tetraploid tumor. The growth rate also increased, as in the other osteosarcomas exhibiting changes in DNA content, indicating that ploidy alterations can be of major significance for tumor behavior.

This investigation shows that human osteosarcomas retain their individual features during serial transplantation in nude mice. However, the changes in ploidy level demonstrate the necessity of continuously monitoring different tumor characteristics of human xenografts. The following studies on the effects of IFN- α , were primarily based on the osteosarcomas in early passages.

Interferon-alpha

Human natural IFN- α has been used clinically as adjuvants to surgical treatment of primary osteosarcoma (Strander 1986). The results of this trial is still inconclusive. Furthermore, nIFN- α treatment of osteosarcoma metastases has been unsuccessful (Caparros et al. 1982). Hence, it remains unclear whether nIFN- α has an antitumor effect on osteosarcoma tumors.

The purpose of these experiments was to investigate the antitumor effects of human nIFN- α on osteosarcomas in nude mice (Brosjö et al. 1985, 1987, 1988a). nIFN- α effects were analyzed by assessment of growth rate, histology, and DNA content. Furthermore, the difference in sensitivity to growth inhibition by nIFN- α , among the 14 osteosarcomas, was related to the analyzed tumor characteristics.

Tumor growth

Dose-response

In two dose-response experiments, using osteosarcomas T3 and T5, 2×10^5 IU of nIFN- α , given as a daily subcutaneous injection, immediately arrested tumor growth. In the experiment with T5, the growth of tumors from mice treated with half of this dose, *i.e.*, 1×10^5 IU/day, was also arrested, but not until 15 days of treatment (Figure 8). A dose of 5×10^4 IU/day had only a slight growth inhibitory effect. In the experiment with T3, only a dose of 2×10^5 IU/day induced growth arrest.

Based on these experiments, 2×10^5 IU/day was chosen as the standard dose in further studies of the sensitivity of osteosarcomas to nIFN- α . The osteosarcomas responded to this standard dose, with tumor regression, growth arrest, or partial growth inhibition, except for T4 which was not affected (Figure 9).

All 14 studied osteosarcomas were growth inhibited by nIFN- α . However, there was a considerable variation in the dose needed to achieve growth inhibition. Tumor regression or growth arrest was seen in 5 of the 14 osteosarcomas with the standard dose 2×10^5 IU/day (Table 5). These 5 tumors (T2, T3, T5, T6, and T10) were regarded as more nIFN- α sensitive and the remaining 9 as less sensitive. Among the latter, 8 were partially growth inhibited and only 1 osteosarcoma was completely insensitive to this dose. The effect of 1×10^6 IU/day, or more, was studied in 7 of these 9

osteosarcomas (Figure 10). These very high doses arrested the growth of 5 and partly inhibited the growth of 2. None was completely insensitive to nIFN- α .

These results show that daily subcutaneous injections of nIFN- α inhibit the growth of human osteosarcomas in nude mice. The individual tumors were dose-response sensitive, *i.e.*, small changes in dose caused major effects on growth. Furthermore, the nIFN- α dose that induced arrest of tumor growth varied considerably among the different osteosarcomas.

Changes in dosage

In an experiment with T2, the effect of 6×10^5 IU nIFN- α , given every third day, was compared to the standard nIFN- α treatment of 2×10^5 IU/day, *i.e.*, the total amount of nIFN- α given was the same. Both dosage regimes reduced growth, but growth arrest was only attained with daily nIFN- α injections (Figure 11).

To investigate whether nIFN- α can induce a permanent change in growth potential, the effect of withdrawal of nIFN- α treatment, or dose reduction, was studied in tumors that had been growth arrested by nIFN- α . In experiments with T2 and T8, tumor growth resumed after withdrawal of nIFN- α treatment (Figure

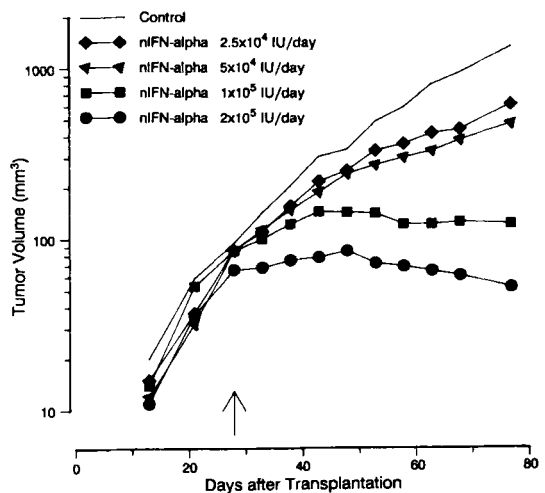


Figure 8. Growth curves of osteosarcoma T5 in nude mice treated with 4 different doses of nIFN- α . Treatment was started at day 28 (arrow).

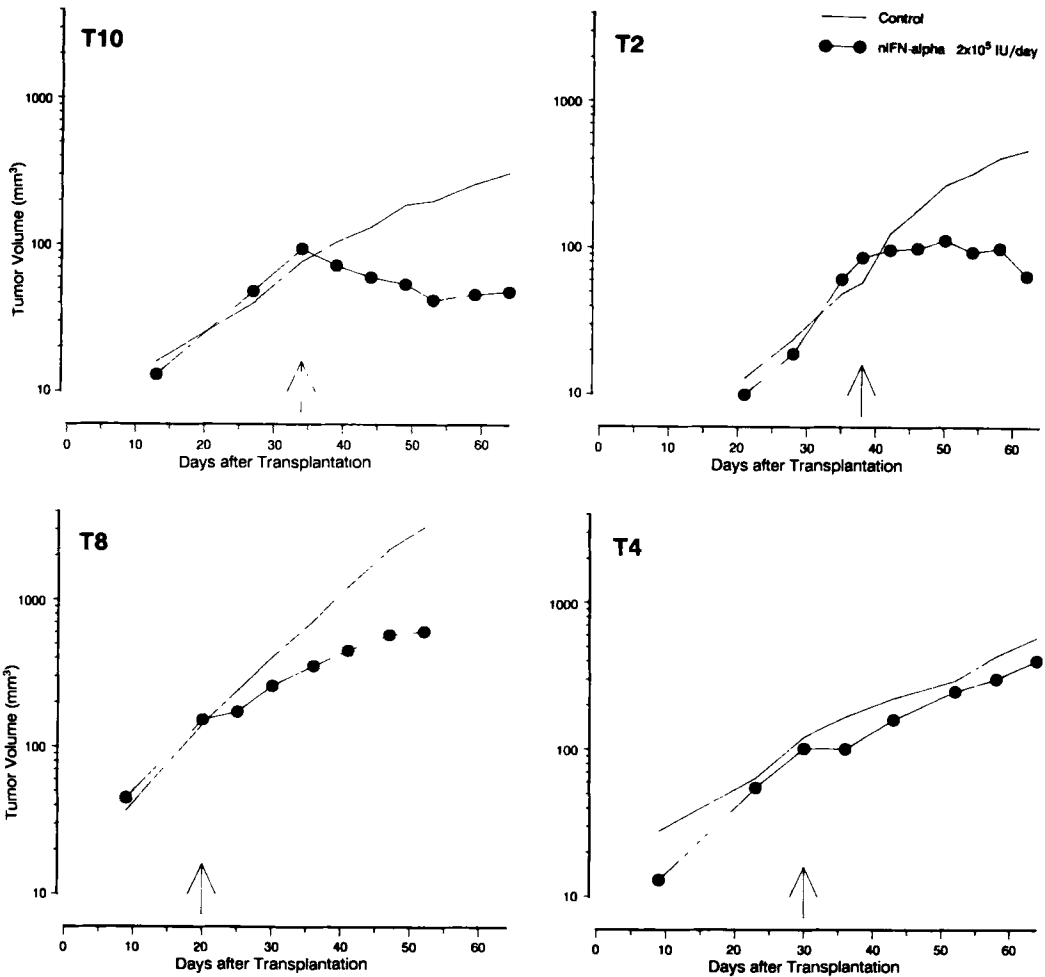


Figure 9. Growth curves of 4 different osteosarcomas in nude mice treated with the standard nIFN- α dose. The arrows indicate start of treatment.

12). In fact, the growth rate became equal to that of tumors in the respective control groups. Similar findings were seen with T4, insensitive to the standard treatment of 2×10^5 IU/day. In this experiment, after an initial period of growth arrest by 1×10^6 IU/day, the daily nIFN- α dose was reduced to 2×10^5 IU in half of the mice whereas the high nIFN- α dose was continued in the other half. Tumor growth resumed immediately upon dose reduction.

From these experiments it is concluded that growth inhibition can only be maintained if nIFN- α is continuously given at the same daily dose. The findings indicate that nIFN- α , in this model, has a cytostatic rather than a cytotoxic antitumor effect.

Sensitivity during serial transplantation

Data from nIFN- α experiments, based on tumors in early and late passage of the same osteosarcoma, were collected to assess whether the sensitivity to nIFN- α changed during serial transplantation. In the 4 osteosarcomas studied in this respect, there was a time period of 9 to 30 months between the 2 nIFN- α experiments compared (Table 6). None became refractory to nIFN- α . The sensitivity to nIFN- α was unchanged in T2 and T5, whereas the growth inhibitory effect was less pronounced in experiments with T7 and T11 in late passage. The ploidy level and growth rate of these 2 latter osteosarcomas had also changed during serial transplantation (page 14).

Table 5. Effect of the standard dose 2×10^5 IU/day nIFN- α on the 14 human osteosarcomas in early passage in nude mice

Tumor	Passage	Treatment length (days)	Treatment group ^a	Number of tumors	Growth Index ^b
T1	5	16	C	9	9.7 3.0
			IFN	8	4.0 1.2***
T2	7	34	C	6	18.4 11.3
			IFN	6	1.0 0.3***
T3	5	90	C	4	33.3 17.2
			IFN	4	1.3 1.0**
T4	6	34	C	4	5.0 1.8
			IFN	4	4.8 3.1
T5	3	60	C	5	27.2 10.3
			IFN	6	1.1 0.4***
T6	5	33	C	4	7.8 2.9
			IFN	4	0.3 0.1***
T7	3	32	C	4	30.1 17.4
			IFN	4	4.3 1.6*
T8	3	31	C	6	15.3 6.1
			IFN	6	5.0 1.8**
T9	3	25	C	7	8.7 2.4
			IFN	6	5.5 2.2*
T10	3	30	C	7	4.4 1.5
			IFN	5	0.5 0.1***
T11	3	26	C	4	13.2 1.8
			IFN	5	2.1 0.7***
T12	3	27	C	6	5.9 1.9
			IFN	7	3.6 1.5*
T13	2	57	C	6	5.1 0.9
			IFN	5	2.2 1.2**
T14	2	29	C	4	9.7 1.0
			IFN	5	5.3 2.2**

^a C control

^b Mean *SD*. Growth Index of treated and the respective control tumors were compared by a 2-tailed Student's *t*-test.

This comparative study indicates that decisive changes in sensitivity to nIFN- α generally do not occur during serial transplantation of human osteosarcomas in nude mice.

Histology

Growth arrest of osteosarcoma xenografts was, in general, accompanied by major histologic changes, suggestive of differentiation and replacement of tumor tissue by stromal host tissue. However, partly growth inhibited tumors showed the same histologic appearance as control tumors. In these tumors nIFN- α treatment was not associated with tumor necrosis nor lymphocytic infiltration, and the Mitotic Indices were unchanged.

In 5 osteosarcomas, T2, T5, T6, T10, and T13, signs of histologic differentiation were evident after nIFN- α induced growth arrest. The tumor tissue was partly or completely replaced by bone and bone marrow of normal appearance (Figure 13). This differentiated bone

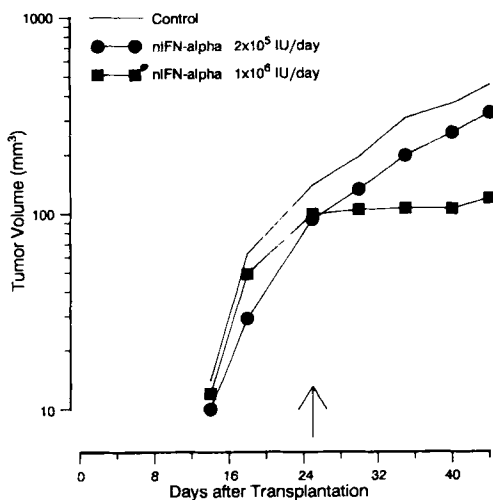


Figure 10. Growth curves of osteosarcoma T4 in nude mice treated with 2 different doses of nIFN- α . Treatment was started at day 25 (arrow).

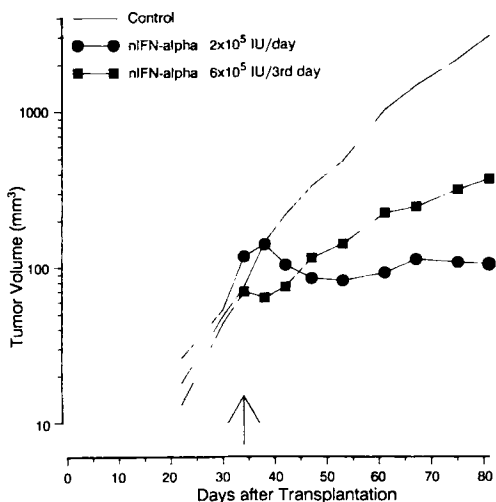


Figure 11. Growth curves of osteosarcoma T2 in nude mice treated daily or every third day, with the same total dose of nIFN- α . Treatment was started at day 34 (arrow).

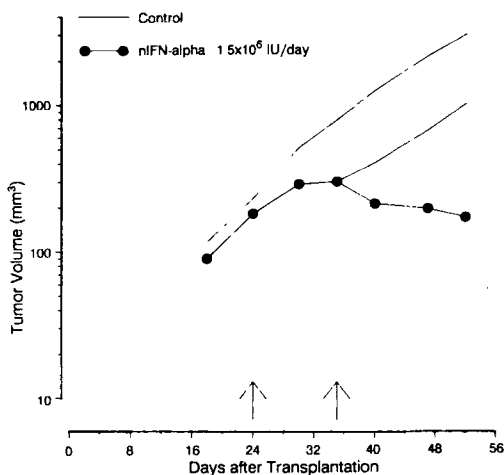


Figure 12. Growth curves of osteosarcoma T8 in nude mice. Treatment was started at day 24 (left arrow). At day 35 (right arrow), treatment was withdrawn from half of the nIFN- α treated mice.

tissue was sometimes seen in the pseudocapsule surrounding the tumor, but more commonly within the tumor. Furthermore, the remaining tumor tissue was heavily mineralized and only a few mitotic figures were seen. In T6 and T10, the tumor tissue was completely replaced by mature bone trabeculae; no osteosarcoma cells were seen. These signs of histologic differentiation were seen in 4 out of 5 tumors that were growth arrested, or reduced in size, by nIFN- α treat-

ment with 2×10^5 IU/day. Normal bone and bone marrow were never seen in the respective controls, and in only 1 (T13) out of the 9 less nIFN- α sensitive osteosarcomas.

Growth arrest of 5 less sensitive osteosarcomas was associated with histologic changes in 2 (T8 and T12). A very high nIFN- α dose of 1.5×10^6 IU/day was needed to growth inhibit these tumors. Histologic examination disclosed lower cell density and an

Table 6. Effect of nIFN- α on 4 human osteosarcomas in early and late passage in nude mice

Tumor	Time between passages (months)	Passage	Dose IU/day ^a	Number of tumors	Volume doubling time (days) ^b
T2	13	7	C	6	9.2 2.6
			2×10^5	6	GA
		13	C	3	8.1 1.7
T5	13	3	2×10^5	7	GA
			C	5	13.4 2.5
		7	C	4	12.2 2.1
			2×10^5	5	GA
T7	9	3	C	3	7.5 1.9
			1×10^6	4	GA
		9	C	6	4.4 0.9
T11	21	3	1×10^6	6	12.3 8.0
			C	4	7.0 0.4
		13	1×10^6	5	GA
			C	8	6.1 0.5
			1×10^6	8	25.1 7.1

^a C control.

^b Mean SD. GA growth arrest.

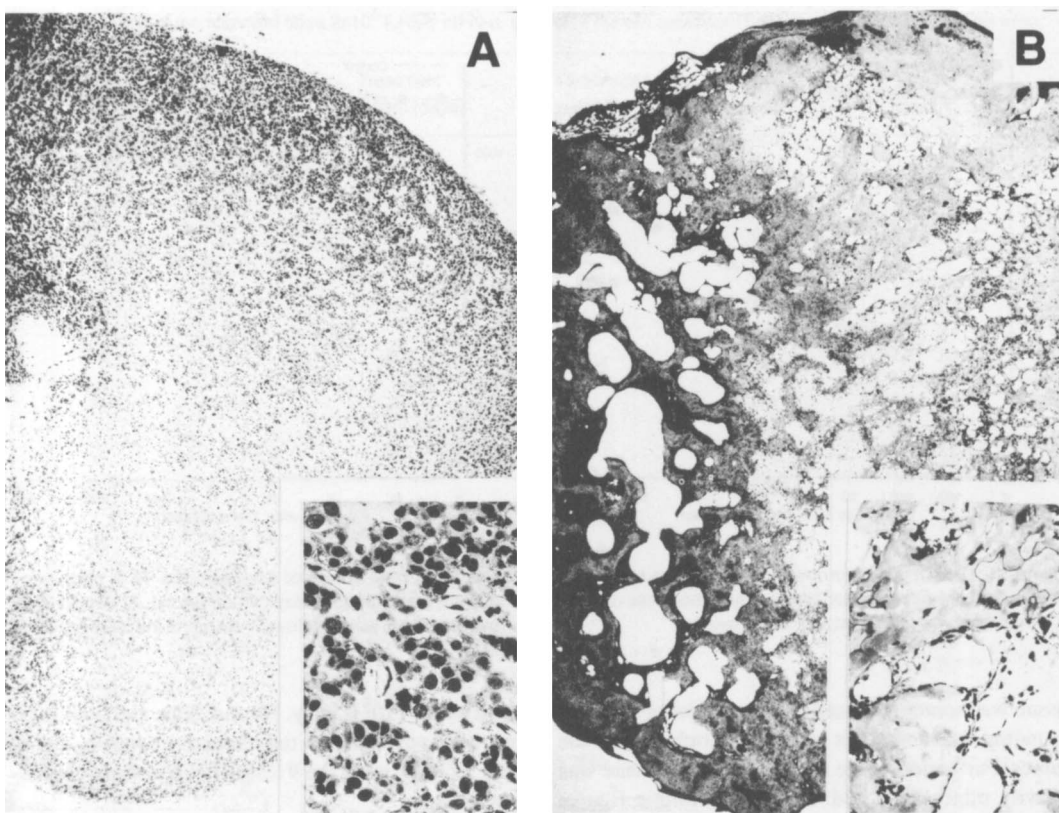


Figure 13. Low power (bar, 500 μm) and high power inset (bar, 45 μm) micrographs of T10, a Grade III chondroblastic osteosarcoma in passage 3 from control (A) and nIFN- α treated (B) mouse. The osteosarcoma tissue was totally replaced by normal bone and bone marrow. Hematoxylin-eosin stain in 1.5 μm sections (Reprinted with permission from Cancer Research).

increased proportion of stroma cells within the tumor. Mitotic activity was significantly decreased. In T8, growth arrested tumors exhibited a mature cartilaginous appearance and the high cellularity of control tumors had largely disappeared. These signs of histologic differentiation were not seen in T12; instead there was areas of central necrosis containing numerous leukocytes.

This study shows that pronounced histologic changes may occur in growth arrested tumors. The finding of histologic differentiation in the more nIFN- α sensitive osteosarcomas implies that tumor growth can not only be controlled by nIFN- α , but osteosarcoma tissue can actually be replaced by normal bone tissue.

DNA analysis

Ploidy level

Flow DNA analysis of tumors from nIFN- α treated mice commonly did not reveal specific changes of the

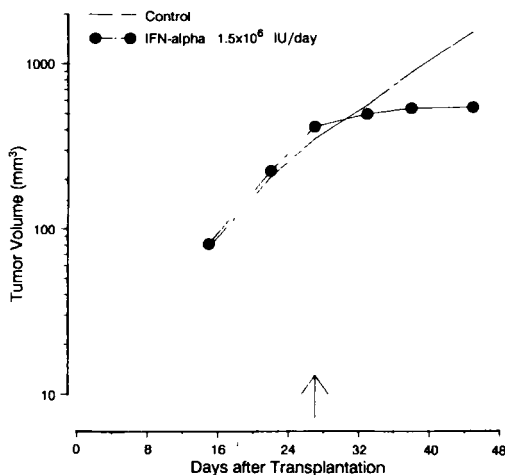


Figure 14. Growth curves of osteosarcoma T12 in nude mice. Treatment was started at day 27 (arrow).

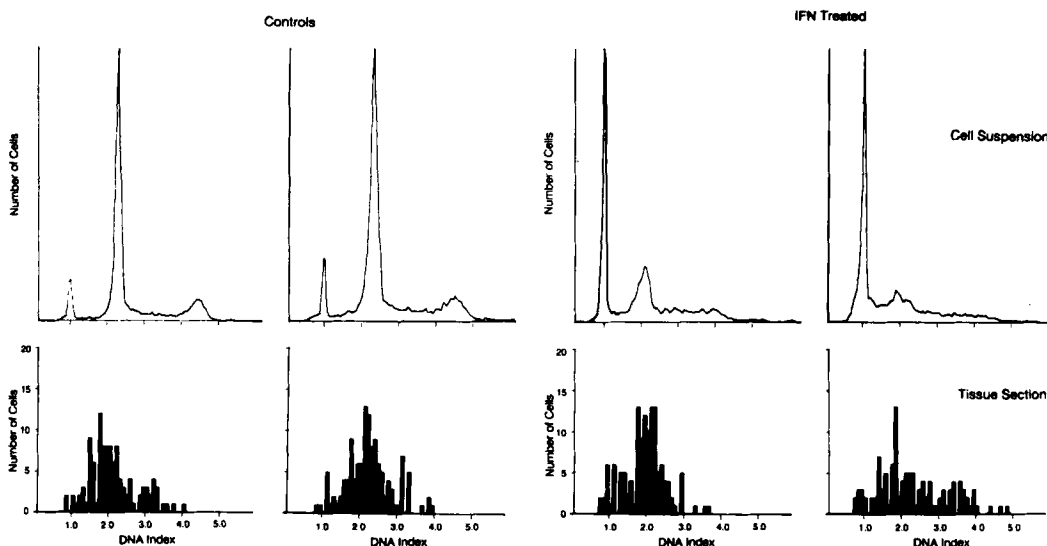


Figure 15. Representative DNA histograms of osteosarcoma T12, obtained by flow cytometry of cell suspension (top) and Image analysis of tissue sections (bottom). Tumors from control (left) and nIFN- α treated (right) mice.

aneuploid DNA content. However, an increase in the proportion of cells with a diploid DNA content was commonly seen. This finding related to the degree of growth inhibition. Hence, analysis of growth arrested tumors exhibiting histologic differentiation (T2, T5, T6, T10, and T13), disclosed that the majority of cells was diploid. In some, a small aneuploid cell population was still evident. Growth arrest of less nIFN- α sensitive osteosarcomas was also associated with an increased proportion of diploid cells.

To analyze this increased proportion of diploid cells, the less nIFN- α sensitive osteosarcomas T8 and T12 were treated by 1.5×10^6 IU/day of nIFN- α . This very high nIFN- α dose arrested the growth of both osteosarcomas (Figure 14). DNA analysis in cell suspensions revealed that this growth arrest was accompanied by a striking reduction in the proportion of aneuploid cells so that the diploid cells became dominant (Figure 15). However, DNA analysis of tissue sections, in histologic areas where osteosarcoma cells predominated, showed that the aneuploid DNA content of the tumors was unchanged.

The combined findings showed that the aneuploid DNA content of osteosarcoma cells was not affected by nIFN- α treatment. However, the decreased proportion of aneuploid cells indicated that nIFN- α treatment reduced the number of osteosarcoma cells within growth arrested tumors.

Cell cycle parameters

Assessment of the proportion of cells in different phases of the cell cycle, based on DNA histograms from growth arrested tumors, was uncertain due to the fact that only a small proportion of aneuploid cells remained after nIFN- α treatment. However, no obvious changes were seen, such as reduction in the proportion of S-phase cells or increased G_0/G_1 -cells. Analysis of tumors partly growth inhibited by nIFN- α , which were not associated with changes of the aneuploid cell proportion, also revealed unchanged cell cycle characteristics.

Thus, nIFN- α treatment of osteosarcomas does not appear to act by blocking cells in a specific phase of the cell cycle. Instead, growth inhibition may result from a decrease in rate of cells proceeding through the cell cycle, or an increased cell loss fraction. Cell cycle analysis, based on DNA histograms, was compatible to both these interpretations.

Tumor features and interferon sensitivity

The aim of this study was to investigate whether sensitivity to growth inhibition by nIFN- α related to specific tumor characteristics. Data regarding growth rate, Mitotic Index and proportion of S-phase cells of untreated control tumors was collected from the nIFN- α experiments of the osteosarcomas in early passage. Assessment of sensitivity to nIFN- α was based on the

Table 7. Proliferative activity and growth rate of control tumors from experiments with the standard nIFN- α dose (Table 5)

Tumor	Number of tumors	Cells in S-phase (%) ^a	Mitotic Index ^a	Volume doubling time (days) ^a
Less IFN sensitive				
T1	9	22 1.3	23 3.8	4.1 0.6
T4	4	28 4.5	40 6.0	11 2.2
T7	4	14 1.9	27 7.6	7.5 1.9
T8	6	25 3.0	26 3.3	7.9 1.0
T9	7	27 2.8	28 3.6	8.8 0.9
T11	4	24 3.6	20 3.2	7.0 0.4
T12	6	20 2.6	25 4.6	9.8 3.0
T13	6	25 4.1	8 2.5	26 2.8
T14	4	30 3.2	Not Done	10 2.9
		24 4.8	25 8.9	10 6.2
More IFN sensitive				
T2	6	10 3.2	13 1.9	9.2 2.6
T3	4	7 2.5	13 1.3	18 3.1
T5	5	19 2.1	20 4.9	13 2.5
T6	4	17 3.0	21 2.5	11 3.1
T10	7	19 4.1	11 4.7	15 3.1
		14 5.5	16 4.6	13 3.4

^a Mean SD. The more IFN sensitive osteosarcomas had lower proportion of S-phase cells ($p < 0.01$) and lower Mitotic Index ($p < 0.05$) compared to the less sensitive (Wilcoxon rank sum test).

growth inhibiting effect of the standard (2×10^5 IU/day) nIFN- α dose (Table 5).

The nIFN- α sensitivity appeared to inversely relate to proliferative activity. Hence, the 5 more sensitive osteosarcomas, *i.e.*, those which were growth arrested at this dose, had a relatively low proliferative activity. The mean S-phase proportion was 14 percent and Mitotic Index 16; whereas the corresponding values for the 9 less sensitive were 24 and 25%, respectively (Table 7). The growth rate of the more nIFN- α sensitive osteosarcomas was not significantly lower compared to the less sensitive. The mean volume doubling times for the 2 groups were 13 and 10 days, respectively.

These findings show that characteristics of the individual osteosarcoma are predictive for the response to nIFN- α treatment. In this respect, the proliferative activity of the tumor cells is more relevant than the growth rate of the tumor.

Comments

IFNs are largely species specific and nude mouse cells can be regarded as insensitive to human IFN. Human IFN does not induce IFN dependent enzymes in nude mice nor stimulate natural killer cells (Balkwill et al.

1982; Uenishi et al. 1983; Heston et al. 1984; Balkwill et al. 1986). However, murine IFN can inhibit the growth of human xenografts in immunodeficient mouse (Gresser et al. 1972; Balkwill et al. 1986). This antitumor effect is considered to reflect a disturbance of human tumor cell and mouse stromal cell interactions. Murine IFN may render the host cells less sensitive to local growth factors, produced by the human tumor, necessary for angiogenesis and stromal cell proliferation (Balkwill et al. 1986).

In the current study, the fact that very high human nIFN- α doses were tolerated by the animals also suggests that mouse cells are insensitive to human IFN. Hence, the antitumor effects were the result of a direct effect on the human osteosarcoma cells. Growth inhibition may be caused by a direct antiproliferative effect on the osteosarcoma cells or, secondarily, by IFN induced changes in the tumor microenvironment (Taylor-Papadimitriou et al. 1985).

The growth of 13 out of the 14 studied osteosarcomas was inhibited, partly or completely, by the standard nIFN- α dose of 2×10^5 IU/day. Assessment of the corresponding dose in humans is dependent on the conversion factor used for comparison. Hence, related to body surface area, 2×10^5 IU in mice corresponds to 20×10^6 IU/m² (Balkwill et al. 1982); a dose which has been given to patients, for at least a limited period of

time (Strander 1986). However, to achieve an equal serum concentration of IFN in humans, as was obtained with 2×10^5 IU in mice, 8×10^6 IU/kg would be needed, *i.e.*, 10 times higher than the maximum tolerated dose. Furthermore, relatively higher serum levels of IFN are achieved in mice compared to humans (Bocci 1985). Hence, the comparison of IFN dosage among different species is of questionable relevance, but it appears that the effective antitumor IFN dose in nude mice was considerably higher than is presently given as adjuvant treatment to osteosarcoma patients (Strander et al. 1988).

Although nIFN- α inhibited the growth of all 14 osteosarcomas, growth arrest was only obtained in 5 with the standard dose 2×10^5 IU/day. In fact, there was more than a ten-fold difference in the dose needed between the most and least sensitive osteosarcomas. Differences in nIFN- α sensitivity appeared to relate to proliferative activity, *i.e.*, osteosarcomas growth arrested by the lower nIFN- α doses had a relatively low proliferative rate. The finding implies that this feature of the individual tumor is of importance for the response to IFN treatment. The relationship between proliferative activity and IFN sensitivity suggests that the less anaplastic tumors, still under a certain growth control, are more susceptible to IFN induced growth inhibition. In clinical trials, the most promising results have been reported from studies of neoplastic entities associated with slow growth and a relatively high degree of differentiation, *i.e.*, carcinoid tumor, hairy cell leukemia, and metastasizing renal cell carcinoma (Quesada et al. 1984; Öberg et al. 1986; Balkwill and Smyth, 1987).

IFN does not act as conventional chemotherapeutic agents. Instead, IFNs are proteins with mechanisms of action similar to hormones, *i.e.*, IFN binds to a specific receptor and a number of effects are induced. Response to nIFN- α treatment should be regarded as the net result of these cellular events. In the present study, complete growth inhibition of the osteosarcomas was often accompanied by a reduced mitotic activity, but not of the S-phase proportion. These combined findings may be interpreted as nIFN- α induces increased cell loss before mitosis and/or decreased rate of cells proceeding through the cell cycle. The concept of increased cell loss being one mechanism of IFN induced growth retardation was introduced by Gresser and Tovey (1978). Hence, increased cell loss fraction may lead to growth inhibition without significantly effecting the growth fraction. Results of cytochemical and histologic analysis supports this interpretation.

However, the precise mechanism of action of IFN- α induced growth inhibition of osteosarcomas needs to be further investigated. Effects on oncogene expression and growthfactors have yet not been studied.

DNA analysis by flow cytometry of growth arrested tumors, revealed that the aneuploid cell population was partly replaced by diploid cells. These tumors exhibited major differences in histologic appearance. The more nIFN- α sensitive osteosarcomas were replaced by bone and marrow tissue of normal appearance, whereas in the less sensitive, growth arrest was accompanied by increased number of stromal cells. The residual tumor tissue, in both groups, looked similar to that of tumors from untreated controls, except that the extracellular matrix became mineralized and there were fewer mitotic figures. Furthermore, DNA analysis in tissue sections showed that the aneuploid DNA content of the remaining tumor cells remained unchanged. These combined findings indicate that IFN induced growth arrest of osteosarcomas leads to the replacement of the tumor cells by normal stromal tissue.

The finding of normal tissue, of the same histogenetic type as the tumor, suggested that the normal bone was produced by differentiated osteosarcoma cells. However, human osteosarcoma tissue contains a bone morphogenetic protein which induces normal mouse mesenchymal cells to differentiate to osteoprogenitor cells (Bauer and Urist 1981; Urist et al. 1983). To investigate whether the bone trabeculae, seen in nIFN- α inhibited tumors, were of mouse or human origin, growth arrested tumors of T2 were studied by immunohistologic techniques (Forster et al. 1988). Applying specific antibodies to human and murine collagen Type I, respectively, revealed that the bone trabeculae were of mouse origin, whereas the remaining tumor tissue was of human origin. Hence, nIFN- α treatment induced an interaction between the tumor and host, resulting in the replacement of the tumor by host derived differentiated bone tissue. In conclusion, growth arrest of osteosarcomas leads to suppression of the tumor cells, but only the more nIFN- α sensitive osteosarcomas had the capacity to induce host normal bone formation. These findings further emphasize the importance of individual tumor characteristics in their response to IFN treatment.

To summarize, all 14 osteosarcomas were growth inhibited by nIFN- α . Since tumors may exhibit a variable sensitivity to different types of IFN (Balkwill et al. 1985), the antitumor effect of IFN- γ was compared to that of IFN- α in the following study.

Interferon-gamma

Combination treatment with IFN- α and IFN- γ is reported to have synergistic antitumor effects (Fleischmann et al. 1984; Koren and Fleischmann 1986). This was mainly based on studies of human and mouse tumor cell lines in vitro, and of mouse tumors in vivo. In studies of human tumors in experimental animals, IFN- γ has only had a weak antitumor effect. (Balkwill et al. 1985; Twentyman et al. 1985; Balkwill et al. 1986).

In the present study, 6 human osteosarcoma xenografts were used to investigate the antitumor effect of IFN- γ , given alone and in combination with IFN- α (Brosjö et al. 1988b). In addition, natural IFN- γ was compared to recombinant IFN- γ .

Pharmacokinetics

After a single subcutaneous injection of 2×10^5 IU of nIFN- γ and rIFN- γ , the highest and also most sustained IFN levels, as determined by both the antiviral assay and the RIA, were obtained with nIFN- γ (Figure 16). The serum levels of nIFN- γ were at least 10 times higher than those of rIFN- γ .

Tumor growth

3 out of the 6 osteosarcomas were growth inhibited by daily subcutaneous IFN- γ injections. T11 and T13 were inhibited by 2×10^5 IU of nIFN- γ (Figure 17), and the same dose of rIFN- γ partially inhibited the growth of T2 (Table 8). A 5 times higher dose of rIFN- γ reduced the growth rate of T11. The growth of the remaining 3 osteosarcomas could not be altered by IFN- γ at the doses given. In the experiment with T12, 4×10^5 IU of nIFN- γ did not inhibit growth (Table 8). Similarly, rIFN- γ was ineffective in T7 (2×10^5 IU) and T8 (1×10^6 IU). For comparison, rIFN- α , given in similar or higher doses as the rIFN- γ , had also no appreciable effect on T7 and T8. However, nIFN- α , as opposed to nIFN- γ , significantly inhibited the growth of T12.

These experiments demonstrate that IFN- γ inhibits the growth of human osteosarcomas in nude mice. The natural type of IFN- γ appeared more effective than the recombinant.

Interferon combinations

The growth inhibitory effect of combined IFN treatment was tested in 3 osteosarcomas. A combination of

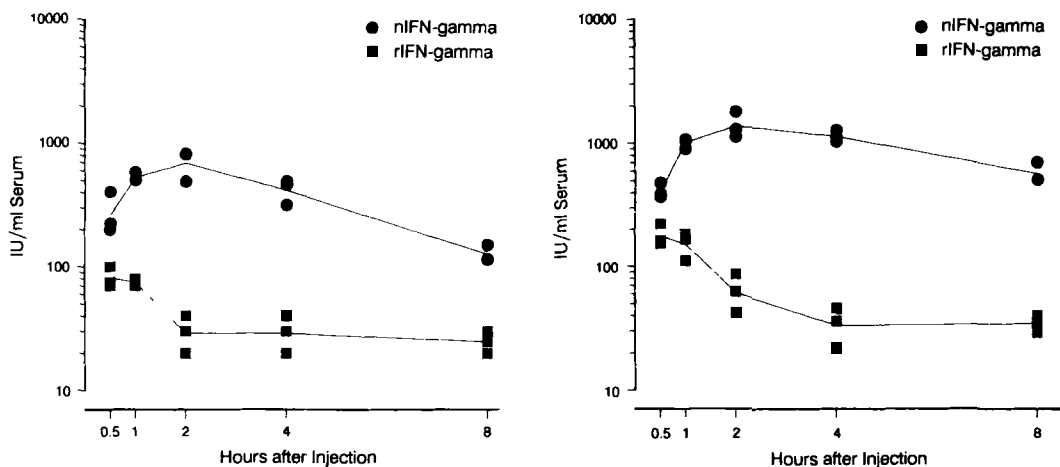


Figure 16. IFN- γ serum concentration, after a single subcutaneous injection of 2×10^5 IU, analyzed by plaque reduction assay (left) and RIA (right). Serum from 3 mice was analyzed at each time point.

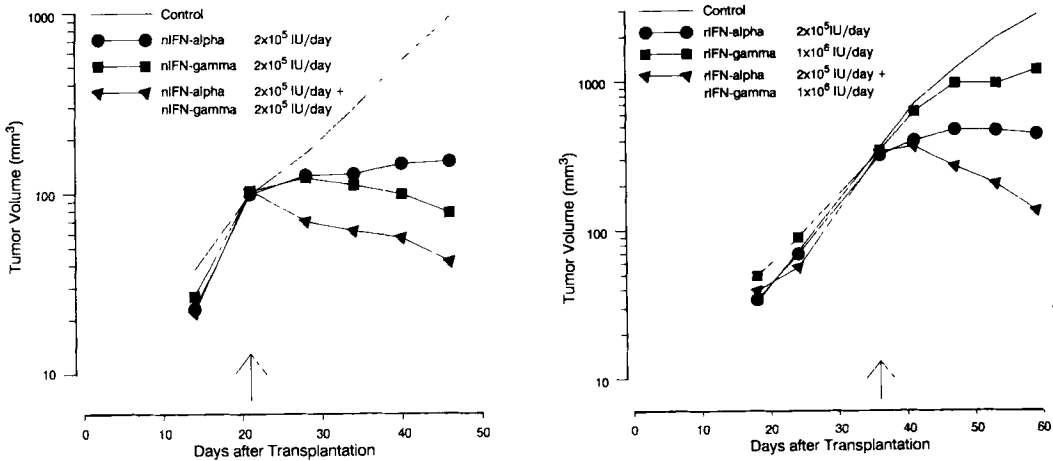


Figure 17. Growth curves of osteosarcoma T11 in nude mice treated with natural IFNs in passage 6 (left); and recombinant IFNs in passage 7 (right). Treatment was started at the arrows.

2×10^5 IU nIFN- α and 2×10^5 IU nIFN- γ induced a significant growth retardation of T12 (Table 8). However, this combination was not more effective than 4×10^5 IU nIFN- α given alone, *i.e.*, the same total IFN dose. No significant reduction of the growth rate of T8 was seen by combining rIFN- α and rIFN- γ .

Regarding T11, combinations of either recombinant or natural IFN- α and IFN- γ resulted in tumor regression (Figure 17). The dose of 2×10^5 IU of nIFN-

α arrested the growth of T11. With the same dose of nIFN- γ , a slight reduction of tumor size was seen towards the end of treatment. Combining these two IFNs, *i.e.*, giving a double total IFN dose, induced tumor regression immediately from the start of treatment. Similar results were obtained with rIFN- α and rIFN- γ . However, a 3 times higher total rIFN dose was needed to achieve a similar growth inhibiting effect as obtained with the nIFNs.

Table 8. Effect of IFN- α and IFN- γ treatment on the osteosarcomas T2, T7, T8, and T12

Tumor	Passage	Treatment length (days)	Treatment IU/day	Number of tumors	Growth Index ^a
T2	17	21	Control	6	2.8 0.8
			2×10^5 rIFN- γ	6	1.8 0.4*
T7	9	13	Control	6	8.6 3.7
			1×10^6 rIFN- α	6	6.7 1.6
			2×10^5 rIFN- γ	6	7.8 2.4
T8	8	19	Control	5	5.3 2.0
			1×10^6 rIFN- α	5	3.8 1.2
			1×10^6 rIFN- γ	5	4.4 0.5
			2×10^5 rIFN- α + 2×10^5 rIFN- γ	6	3.7 1.8
T12	4	18	Control	5	3.6 1.3
			4×10^5 nIFN- α	6	1.5 0.4**
			4×10^5 nIFN- γ	6	3.6 1.0
			2×10^5 nIFN- α + 2×10^5 nIFN- γ	6	2.0 0.5*

^a Mean SD. Growth Index of treated and control tumors were compared by a 2-tailed Student's *t* test.

Table 9. Effect of IFN- α and IFN- γ treatment on the osteosarcomas T11 and T13

Tumor	Passage	Treatment length (days)	Treatment (IU/day)	Number of tumors	Growth Index ^a	Mitotic Index ^b
T11	6	32	Control	6	13.2 4.2	18 15-20
			2x10 ⁵ nIFN- α	5	1.6 0.2***	14 8-17
			2x10 ⁵ nIFN- γ	6	0.5 0.1***	4 2-6
			2x10 ⁵ nIFN- α + 2x10 ⁵ nIFN- γ	6	0.2 0.1***	2 0-4
			Control	6	7.8 1.1	22 19-25
	7	24	2x10 ⁵ rIFN- α	4	1.5 0.3***	11 8-15
			1x10 ⁶ rIFN- γ	5	4.2 2.6*	9 5-11
			2x10 ⁵ rIFN- α + 1x10 ⁶ rIFN- γ	6	0.4 0.4***	1 0-1
			Control	6	5.1 0.9	8 5-11
			2x10 ⁵ nIFN- α	5	2.2 1.2**	4 0-14
T13	2	57	2x10 ⁵ nIFN- γ	5	1.5 0.8***	1 0-1

^a Mean SD. Growth Index of treated and control tumors were compared by a 2-tailed Student's *t*-test.

^b Mean and range.

In conclusion, nIFN- γ appears to have similar anti-tumor properties as IFN- α . An additive growth inhibitory effect was seen by combining IFN- α with IFN- γ .

Histology

Growth inhibition of T11 and T13, was accompanied with major morphologic changes. Examination of tumors from IFN treated mice, disclosed that the number of mitoses decreased with increasing growth inhibition (Table 9). In fact, almost no mitoses were seen in tumors which decreased in size during IFN treatment. Furthermore, in 4 out of 10 tumors of the osteosarcoma T13, the tumor was totally replaced by bone trabeculae and bone marrow, *i.e.*, normal bone tissue. These 4 tumors all diminished in size during IFN treatment; 3 tumors were from mice treated by IFN- γ and 1 by IFN- α .

This proves that the phenomenon of bone induction by growth inhibited osteosarcomas is not specific for IFN- α treatment, but can also be elicited by IFN- γ .

Comments

In previous studies, based on the nude mouse model, IFN- γ had no, or only slight, antitumor effect on human lung, bowel, or breast carcinomas (Balkwill et al. 1985; Twentyman et al. 1985; Balkwill et al. 1986). In these experiments, only the recombinant type of IFN- γ was used, given as a daily intraperitoneal or subcutaneous injection of 2-4 x 10⁵ IU. As in these studies,

the same dose of rIFN- γ was ineffective in 3 out of the 4 tested osteosarcomas. However, T2, previously shown to be sensitive to nIFN- α , was inhibited by rIFN- γ .

The poor antitumor effect of subcutaneously administered rIFN- γ can probably be due to low serum concentrations in nude mice. Balkwill and co-workers (1985) have shown that intraperitoneal injections of rIFN- γ led to 10 times higher serum levels than those obtained by the subcutaneous route. In the present study, subcutaneously administered nIFN- γ gave ten times higher serum levels as compared to rIFN- γ . The reason for low serum levels after subcutaneous injections of rIFN- γ is not known, but may be due to the fact that recombinant, as opposed to natural, IFN- γ is not glycosylated (Gray et al. 1982). Differences in pharmacokinetics may thus be responsible for the finding that 5 times higher dose of rIFN- γ , as compared to nIFN- γ , was needed to achieve antitumor effect.

In the present study, an additive effect was seen with natural or recombinant IFN combinations. The finding of tumor regression in T11, would even suggest synergism. However, in the experiment with T12, nIFN- α was equally effective as the combination of nIFN- α and nIFN- γ . Finally, the lack of effect of combination treatment in T8 argue against synergism. Possibly, combinations of IFN- α and IFN- γ may be advantageous, compared to single IFN treatment, in certain tumors, whereas, in others, treatment with only one type of IFN may be preferable.

In the present study, histologic differentiation were seen after treatment of T13 with nIFN- γ . The reduced mitotic rate, after IFN treatment of T11 and T13, sug-

gests an antiproliferative effect. However, the histologic changes observed were more related to the tumor studied and degree of growth inhibition, rather than to the type(s) of IFN given.

In summary, IFN- γ inhibits the growth of human osteosarcomas in nude mice. This effect was seen with both natural and recombinant IFN- γ , although lower doses of the natural type were needed, reflecting dif-

ferences in pharmacokinetics. The antitumor effect of nIFN- γ was equal to that of IFN- α . Combination treatment with the two IFN types resulted in an additive, but not synergistic, effect. The sensitivity of the individual osteosarcoma to growth inhibition by IFN- α or IFN- γ , respectively, appears to be the most important factor determining the antitumor response.

Summary

This work assessed the transplantability of human osteosarcomas to immunodeficient nude mice. Osteosarcomas serially transplanted in nude mice were characterized by growth rate, histologic features, and nuclear DNA content. The osteosarcoma xenografts were used to investigate the antitumor effects of interferon (IFN).

Tumor tissue from 25 primary osteosarcomas was transplanted into nude mice. All tumors were histologically of high grade (III-IV). Flow DNA cytometry disclosed that all, except 1, had a nondiploid DNA content. 14 of the 25 osteosarcomas grew in serial passage in nude mice, i.e., the take rate was 0.6. The transplantable osteosarcoma group was characterized by a predominance of Grade IV lesions, and a high proportion of proliferating cells, compared to the nontransplantable.

The 14 osteosarcoma xenografts, established in nude mice, were heterogeneous with respect to growth rate, histologic subtype, DNA content, and proliferative activity. However, the osteosarcomas retained the basic characteristics of their respective original tumor; the xenografts exhibited the same histologic appearance and DNA content in the first 2 passages in nude mice.

During serial transplantation of the 14 osteosarcomas, the histologic features remained unaltered from passage to passage during the observation period of up to 3 years. The aneuploid DNA content was also unchanged over time in most tumors. However, in 4 osteosarcomas with 2 aneuploid cell populations, the cell population with the higher DNA content became predominant, while the other gradually disappeared. Hence, the changes in DNA content involved polyploidization, followed by selection of the cell population with higher DNA content. At the same time growth rate increased, but histologic features were unchanged. This study of osteosarcoma, serially transplanted in nude mice, shows that growth rate, histologic appearance, and DNA content are relatively stable tumor features. The observed changes in DNA content illustrate the development of aneuploidy in malignant tumors.

The antitumor effects of human nIFN- α were assessed in the 14 osteosarcoma xenografts. In dose-response experiments, based on 2 different tumors, 2×10^5 IU/day of nIFN- α was found to arrest tumor

growth. This dose was chosen as the standard dose in subsequent experiments. Among the 14 osteosarcomas, tumor regression or growth arrest was seen in 5, whereas 8 were only partially growth inhibited with the standard dose. The remaining osteosarcoma was growth inhibited with higher nIFN- α doses. Hence, all 14 analyzed osteosarcomas were sensitive to the antitumor effect of nIFN- α . The lowest dose needed to induce growth arrest varied considerably among the different osteosarcomas. Tumor growth resumed when nIFN- α treatment was discontinued or when the dose was reduced.

The 14 osteosarcomas were divided into 2 groups, based on the growth inhibitory effect of the standard nIFN- α dose (2×10^5 IU/day). The group of 5 osteosarcomas, growth arrested by this dose, had lower proliferative activity as compared to the group of 9 less nIFN- α sensitive tumors. Hence, this characteristic of the individual osteosarcoma was predictive for the response to nIFN- α treatment.

DNA analysis in tissue sections showed that the aneuploid DNA content was unchanged in nIFN- α treated osteosarcoma cells. However, flow cytometry in cell suspensions of growth arrested tumors disclosed that the proportion of diploid cells increased. This finding implies that nIFN- α treatment induced depletion of the aneuploid tumor cells. Histologically, these growth arrested tumors also exhibited changes. In less nIFN- α sensitive osteosarcomas, an increased number of leukocytes and stromal cells was seen and there was also more extracellular matrix. On the other hand, the more nIFN- α sensitive osteosarcomas were partly or completely replaced by bone trabeculae and hematopoietic tissue. This finding probably represented the result of host and tumor cell interaction. Hence, nIFN- α inhibited tumors induced the host stromal cells to differentiate into normal osteogenetic tissue, replacing tumor tissue.

The antitumor effects of IFN- γ were investigated in 6 osteosarcomas. nIFN- γ appeared to have similar antitumor properties as IFN- α . However, rIFN- γ was less effective than nIFN- γ , apparently due to differences in pharmacokinetics; subcutaneously administered nIFN- γ gave 10 times higher serum levels than the recombinant type. Combination treatment with IFN- α and IFN- γ induced an additive, but not synergistic, growth inhibitory effect in 2 osteosarcomas, whereas 1 was

insensitive to IFN combination. Hence, previous reports of synergism between IFN- α and IFN- γ could not be confirmed by these experiments. The IFN sensitivity of the individual osteosarcoma was more important, for the antitumor response, than the type of IFN used.

In summary, human osteosarcomas transplanted to nude mice exhibited a high take rate compared to other tumor entities, probably reflecting the highly malignant nature of osteosarcoma. The histologic appearance and aneuploid DNA content of the original tumors was retained during serial transplantation, although polyploidization of tumor cell populations occurred. All 14 studied osteosarcomas were growth inhibited by nIFN- α treatment, but the dose sensitivity varied considerably among the tumors. Osteosarcomas exhibiting relatively low proliferative activity were the most sensitive to nIFN- α . Employing this tumor model, nIFN- γ had similar antitumor properties as IFN- α and combination IFN treatment did not appear advantageous.

Regarding the clinical significance of these experiments, the most important finding was that all studied osteosarcomas were sensitive to the antitumor effect of IFN. The doses needed to inhibit tumor growth were generally higher than those tolerated by humans and it is difficult to transfer data obtained from experiments on nude mice to the clinical studies. However, the findings imply that IFN would be an effective antitumor agent in osteosarcoma, provided the morbidity associated with systemic treatment can be reduced. IFN treatment has to be given for an extended period of time, but it remains unclear whether IFN can completely eradicate osteosarcoma tumors. However, growth arrested tumors were replaced by host stromal tissue, and the proportion of tumor cells was reduced, showing that the antitumor effect of IFN was more pronounced than mere measurement of tumor size would suggest. The sensitivity of osteosarcoma to IFN was inversely related to the tumor cells proliferative activity. By improved methods of assessing proliferative rate of original tumors it may prove feasible to select patients responding to IFN treatment.

Acknowledgments

I wish to express my sincere gratitude to my friends Olle Nilsson and Henrik Bauer.

This study was supported by grants from the Jonas Björnberg Memorial Foundation, IngBritt and Arne Lundberg's Research Foundation, the Cancer Society of Stockholm, the Swedish Cancer Society, and the Karolinska Institute's Funds.

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