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Characterization of Treatment Response to Recombinant Interferon-α2b in Osteosarcoma Xenografts

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Abstract

Interferons (IFNs) may target cancer cells both through their regulation of the immune response, effect on angiogenesis and through direct effects on cancer cells. Treatment response has been demonstrated in osteosarcoma patients, but tumour resistance to IFN-α is common. Hence, understanding the molecular mechanisms involved in response and resistance is essential for improving therapeutic efficacy. Of five xenografts screened for specific growth delay in response to treatment with unconjugated and PEGylated IFN-α2b, one displayed growth inhibition and tumour shrinkage. Growth inhibition increased on a dosing schedule of PEGylated IFN every third day. Xenografts resistant to PEGylated IFN were similarly resistant to unconjugated IFN. Combination treatment with PEGylated IFN and doxorubicin resulted in improved growth control rates. Transcriptional profiling analysis of the one sensitive and two resistant xenografts identified a common set of 79 genes significantly affected by IFN- α2b treatment independent of tumour growth inhibition. All but four of the 79 genes were up-regulated. The majority of these genes were well characterized IFN-stimulated genes and core members of the IFN-α signalling pathway. The expression of a set of 128 unique genes changed only in the sensitive xenograft; 52/128 genes were up-regulated. The specific gene expression pattern seen in the responsive xenograft identified possible pathways important for the antitumour effect of IFN-α in osteosarcoma, including subsets of genes involved in cell adhesion and osteogenic tissue development. The observed improved control rates of combined treatment with IFN and doxorubicin are encouraging and should be further explored.

Keywords: Osteosarcoma; Xenograft; Interferon; Gene expression profiling

Abbreviations: IFN: Interferon; ISG: Interferon Stimulated Gene; ISRE: IFN-Stimulated Response Elements; OS: Osteosarcoma; PEGylation: Conjugated to Poly-Ethylene-Glycol

Background

Osteosarcoma (OS) is the most frequent primary malignant tumour of the skeletal system in children and young adolescents. Although a multidisciplinary approach with specialized surgery and combination chemotherapy has led to significant survival improvement during the last three decades, a relapse rate of 30-40% within five years remains (Souhami et al., 1997; Bacci et al., 2000; Bielack et al., 2002; Smeland et al., 2003; Ferrari et al., 2005). Further improved survival appears to depend on the optimal integration of novel drugs into the existing treatment protocols. Interferons (IFNs) are biological response modifiers that may inhibit cancer cell growth by regulation of the immune response, inhibition of angiogenesis and direct antitumour activity (Balkwill, 1985; Gresser, 1989). By interactions with their specific cell surface receptors IFNs activate signal transducer and activator of transcription (STAT) complexes and initiate the classical Janus kinase-STAT (JAK-STAT) signalling pathway (Fu et al., 1992; Schindler et al., 1992). Activated STATs form complexes that bind to specific IFN-stimulated response elements (ISRE) or IFN-γ-activated site (GAS) elements within promoters of interferon stimulated genes (ISGs) to initiate transcription. In addition, non-classical signaling (involving MAPK, PI3K and NF-κB) is necessary to explain the full extent of the transcriptional response (Platanias, 2005; Du et al., 2007). IFN-α subtypes differ in their binding affinities to the IFN receptor and also have variable efficacy on tumour growth (Balkwill, 1985; Foster and Finter, 1998; Yamaoka et al., 1999). Whereas the early studies were performed with mixtures of IFNs extracted from white blood cells, most clinical trials have been performed with single-species recombinant IFN-α, and more recently with variants conjugated to poly-ethylene-glycol (PEGylation). PEGylation delays the elimination of IFN, allowing for once weekly treatment in humans (Glue et al., 2000), and carries a promise of higher efficacy and reduced toxicity (Eggermont et al., 2008). Direct antitumour activity in osteosarcoma has been demonstrated *in vitro* (Strander and Einhorn, 1977; Dannecker et al., 1985) and *in vivo* (Masuda et al., 1983; Hofmann et al., 1985; Brosjo, 1989). Pioneering work at the Karolinska Hospital in Stockholm strongly indicated single agent activity of adjuvant human IFN-α in patients with non-metastatic high-grade osteosarcoma (Muller et al., 2005;

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Strander, 2007). No clear benefit of IFN-β was observed in the only completed randomized trial testing the additional effect of IFN following conventional chemotherapy (Winkler et al., 1984). EURAMOS1, a major randomized OS trial (www.euramos.org) explores sequential maintenance treatment with PEGylated IFN-α2b for the favourable prognostic subgroup with good histologic response to neoadjuvant chemotherapy with cisplatinum, doxorubicin and high-dose methotrexate. Although IFN is in some clinical use in advanced osteosarcoma, the relative contributions of direct antitumour effects, inhibition of angiogenesis and indirect immune-mediated effects are not known. Resistance to IFN is frequently seen, and an understanding of the molecular mechanisms involved in response and resistance is essential for improving the therapeutic efficacy. Furthermore, IFN-α has been effective in the treatment of multidrug resistant osteosarcoma cell lines (Manara et al., 2004) and may be a way to overcome chemotherapy resistance. We therefore investigated the growth response of PEGylated IFN-α2b and unconjugated IFN-α2b treatment and the effect of PEGylated IFN-α2b on gene expression in osteosarcoma tissue using human xenograft models. Finally, aiming at integrated bio-chemotherapy, we extended our experimental series by combining IFN treatment with doxorubicin, which is one of the four active drugs in OS treatment (Blaney et al., 1993).

Material and Methods

Animals

Female athymic mice (Balb/c: nu/nu) were bred in our animal facility, weaned after 21 days and maintained in a pathogen-free environment at controlled temperature (21 +/- 0.5°C) and humidity (55-65%) on a 12 hour light cycle. Sentinels were tested according to FELASA's health monitoring recommendations. Groups of up to eight mice were kept in transparent polycarbonate cages (Tecniplast Eurostandard type III, Scanbur BK, Nittedal, Norway) on aspen chip bedding (B&K Universal, Hull, UK) with pellet feed (RM3, Special Diets Services, Witham, UK) and acidified water supplied *ad libitum*. Morbidity was controlled for by daily inspections focusing on behaviour, posture or weight loss. Animals were sacrificed for ethical reasons in case of weight loss >10% or tumour diameter >20 mm. Before transplantation or sacrifice by cervical dislocation mice were anaesthetised with intraperitoneal injection of 0.1 mg/kg fentanyl, 5 mg/kg fluanison (Janssen Pharmaceutica, Beerse, Belgium), and 2.5 mg/kg midazolam (Roche, Basel, Switzerland). All procedures involving animals were performed according to protocols approved by the National Research Authority in compliance with the European Convention for the Protection of Vertebrates Used for Scientific Purposes.

Xenografts

Tumour fragments were sampled from adolescent high grade osteosarcomas, four from primary tumours (TSx, KPBx, OHSx, MPAx) and one from a pulmonary metachronous metastasis (HPBx). Tumour tissue was implanted in the flanks of nude mice and propagated by serial transplantation (Bruheim et al., 2004). The mice were allocated to treatment groups when tumours reached 50-70 µl. Tumours were measured twice weekly and tumour volumes calculated as 0.5 x length x width^2. Relative tumour volume (RTV) was calculated for each individual tumour for a specific number of days after start of treatment: RTV = $Volume_{day\ X}$ *100 / $Volume_{day\ 0}$. Tumour doubling time (TD) was defined as the time from the start of treatment to the first doubling of the median RTV. The drug effect was expressed as specific growth delay SGD = ($TD_{treated}$ – $TD_{control}$)/ $TD_{control}$ and treated to control rate T/C (%) = $RTV_{treated}$ x 100 / $RTV_{control}$. Based on earlier experience SGD of > 1.0 and T/C of < 50% were defined as antitumour activity (Bruheim et al., 2004).

Radio-labelling of PEGylated IFN

To estimate elimination half life in mouse serum, PEGylated IFN was radio-labelled with ^{125}I. For this analysis, PEGylated IFN was radio-labelled with ^{125}I at an equimolar ratio. An Iodogen tube (Pierce) was prewashed with 1 ml of a pH 7.5 iodination buffer (25 mM Tris, 0.4 M NaCl, all Sigma). 30 µl iodination buffer and 20 MBq ^{125}I dissolved in 7 µl water was added to the iodination tube and incubated for 5 min under continuous shaking, transferred to a tube containing 50 µg interferon-α in 50 µl water and incubated for another 5 min. Iodination was terminated by adding 50 µl of iodination buffer with 10 mg/ml of tyrosine (Sigma). Finally free and bound iodine were separated on a column (Paus et al., 1982). Eight mice were randomly assigned to two groups. Mice were pre-treated with potassium iodide 10 µg/day in the drinking water for 5 days before subcutaneous injection with ^{125}I-labeled PEGylated IFN at 10 and 100 µg/kg (0.3 and 3 MBq). Each mouse was sampled daily for 20-40 µl blood with a heparinised microcapillary (capillary tubes for microhematokrit, art. Nr. 110690, Kebo-Lab Stockholm) (Hem et al., 1998). At the indicated time points (24 h, 7 days) mice were anesthetized, cardially aspirated and killed before sampling tumour-tissue and hind leg muscle. Serum was spun for 7 minutes at 12 800 rpm in a Hematokrit 24 centrifuge (Hettich Zentrifugen), diluted in PBS, and tissue samples were minced in PBS before filtration on a NAP-5 column (Pharmacia Biotech) equilibrated with PBS, and the radioactivity was counted in a Wallac automatic gamma counter (Perkin Elmer Life Science) together with samples of known activity. We found excellent correlation between the capillary plasma and cardial serum measurements (r = 0.99).

Drugs

Unconjugated IFN-α2b (Intron A, Schering Plough, Oslo, Norway, specific activity 2.6x10^8 IU/mg protein) was administered using a prefilled injection pen. MonoPEGylated IFN-α2b (PegIntron, Schering Plough, specific activity 6.4x10^7 IU/mg protein) was reconstituted in water. Stock solutions of diluted PEGylated IFN were stored for a maximum of 2 weeks at -80°C. Immediately before use the stock was diluted with PBS + BSA (Sanceau et al., 2002) to the final concentration in a volume of 0.1 ml. IFNs were injected subcutaneously (s.c.) to the lower back of the mice. Control mice were treated with s.c. PBS + BSA. Doxorubicin (Adriamycin, Pharmacia Upjohn, Stockholm, Sweden) was dissolved in physiological saline and administered intravenously (i.v.). Unconjugated IFN was administered at daily doses of 30,000, 300,000 or 1 million IU/mouse (equivalent to weekly doses of 40, 400 or 1,350 µg/kg). PEGylated IFN was tested at weekly doses of 10, 100 or 300 µg/kg or every third day at an equivalent dose of 4.3, 43 or 129 µg/kg. The total treatment interval for IFN was 3 weeks. Combined treatment with IFN and doxorubicin was started with 10 or 100

µg/kg/week PEGylated IFN followed 24 hours later by 8 mg/kg doxorubicin equalling the maximal tolerated dose (Bruheim et al., 2004); the doxorubicin injection was repeated once after a week. For microarray experiments mice were treated with 100 µg/kg PEGylated IFN and the tissue were sampled both 24 and 48 hours later. In the combination group, mice were treated with 100 µg/kg PEGylated IFN, followed 24 hours later by 8 mg/kg doxorubicin. Tissue was sampled 24 hours after the doxorubicin treatment.

Gene expression microarray analysis

RNA isolation, labelling and microarray hybridizations: Tissue samples were snap frozen on liquid nitrogen and stored at -80°C until use. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and stored at -80°C. Samples were quantified on a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and RNA integrity was determined on Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). RNA from several animals was pooled. In the initial experiments hybridizations were done on cDNA arrays printed at the core facility of the Norwegian Microarray Consortium at the Norwegian Radium Hospital (Oslo, Norway) as described earlier (Prasmickaite et al., 2006). For the final experiments amplification and labelling of 500 ng RNA from treated and untreated TSx, MPAx and HPBx xenografts was performed using the Illumina TotalPrep RNA amplification kit (Illumina Inc., San Diego, CA, USA). The microarray experiments were done using the Whole-Genome Gene Expression Direct Hybridization Assay and Illumina Human-6 Expression BeadChips version 2 arrays (Illumina Inc.) consisting of >48,000 different probes represented with an average 30-fold redundancy across the array. Technical replicates were hybridized for all samples. All laboratory processing and hybridisations were performed according to manufacturer's protocols.

Microarray pre-processing and analysis: The slides were scanned with the BeadArray Reader (Illumina, Inc.) and data extraction and initial quality control of the bead summary raw data were performed using BeadStudio (version 3.1.3.0) from Illumina and the Gene Expression module 3.2.6. Additional quality control before and after quantile normalization and pre-processing was performed with the R package (Du et al., 2007; Du et al., 2008; Lin et al., 2008) which is a part of the Bioconductor project (R-Development-Core-Team, 2009) and the data was annotated using the HumanWG-6_V2_R4_11223189_A annotation file from Illumina. Changes in gene expression were detected at the probe level with one gene represented by one or several probes. To find probes that were differentially expressed between IFN treated and untreated samples for each of the xenografts, we applied linear models for microarray data (Limma) significance analysis with a moderated t-statistic using a simple Bayesian model (Smyth, 2004). The cut-off value for log2-fold-change was set to 0.57 (fold-change >1.5) and the p-value was adjusted for multiple testing by Benjamini and Hochberg's method (Benjamini and Hochberg, 1995) with restriction $p < 0.01$. When identifying probes related to IFN resistance, the difference in expression levels between the sensitive and each of the resistant xenografts had to be at least 1.5 fold. The discriminatory gene lists were further analysed in the online Database for Annotation, Visualization and Integrated Discovery (DAVID, 2008) (Dennis et al., 2003) to organize the genes and identify enrichment based on common functional features in KEGG biochemical pathways and Panther ontologies Biological Process and Molecular Function. To measure the gene-enrichment in annotation terms the number of genes on the differentially expressed lists were compared to all the genes on the Illumina array. The significance of enrichment was indicated by p-values calculated as EASE score (Hosack et al., 2003), a modified Fisher Exact P-Value for gene enrichment analysis, and the cut-off was set to $p < 0.01$. The data was submitted to ArrayExpress with accession E-TABM-707. Interferome, a recently published database of ISGs and putative ISRE in the promoters of these genes (Samarajiwa et al., 2008) was used to investigate IFN signatures in the gene lists of differentially expressed genes.

Results

Treatment response of the xenografts

Of a total of five osteosarcoma xenografts screened for growth inhibition in response to treatment with PEGylated IFN-α2b, only one (HPBx) was found to be sensitive (Figure 1). Xenografts resistant to PEGylated IFN were also resistant to treatment with unconjugated IFN (Figure 1G, Figure 1H). In the sensitive xenograft (HPBx), weekly administration of 100 µg/kg PEGylated IFN gave a weak response (SGD of 0.25 and T/C of 69%; Figure 1C). Due to strong growth inhibition of unconjugated IFN in the same xenograft (Figure 1A), we examined the half life of PEGylated IFN in our model. Iodination of PEGylated IFN indicated a half-life of 15-16 hours in nude mice, compared to 27-39 hours in humans (Glue et al., 2000) (Figure 2). An adapted treatment schedule of PEGylated IFN every third day at a dose equivalent of 100 and 300 µg/kg/week improved efficacy compared to weekly administrations and strongly suppressed tumour growth (SGD of 1.25 and 7.5; T/C of 15% and 5%; Figure 1B). Daily treatment with unconjugated IFN at a dose equivalent of 40µg/kg/week (30,000 IU/mouse/day) was less effective (specific growth delay of 0.75 and a T/C of 30%) whereas a dose of 400µg/kg/week (300,000 IU/mouse/day) for 21 days prevented any regrowth of the sensitive xenograft for a period of at least 90 days after end of treatment. We observed a dose-response relationship for both agents (Figure 1B).

Combined treatment with doxorubicin and PEGylated IFN: Pilot experiments were performed examining the potential of concomitant treatment of the sensitive xenograft (HPBx) with IFN and doxorubicin. When the IFN-sensitive xenograft HPBx was treated with 8 mg/kg doxorubicin as monotherapy a moderate growth delay was obtained (SGD of 1.25, T/C of 44%). Treatment with PEGylated IFN followed by doxorubicin 24 hours later was considerably more effective than monotherapy with either doxorubicin or IFN (SGD up to 2.5, T/C of 26%, Figure 1D).

Response in gene expression

Initial analyses of time course and dose response: Initial experiments were performed to investigate the time course and dose-dependence of the response to PEGylated IFN (Figure 3). Known ISGs were among the strongest induced, and showed highest expression levels with the highest dose and peaked within 12-24 hours. Based on these initial results we selected a dose of 100 µg/kg PEGylated IFN for 24 hours for further experiments

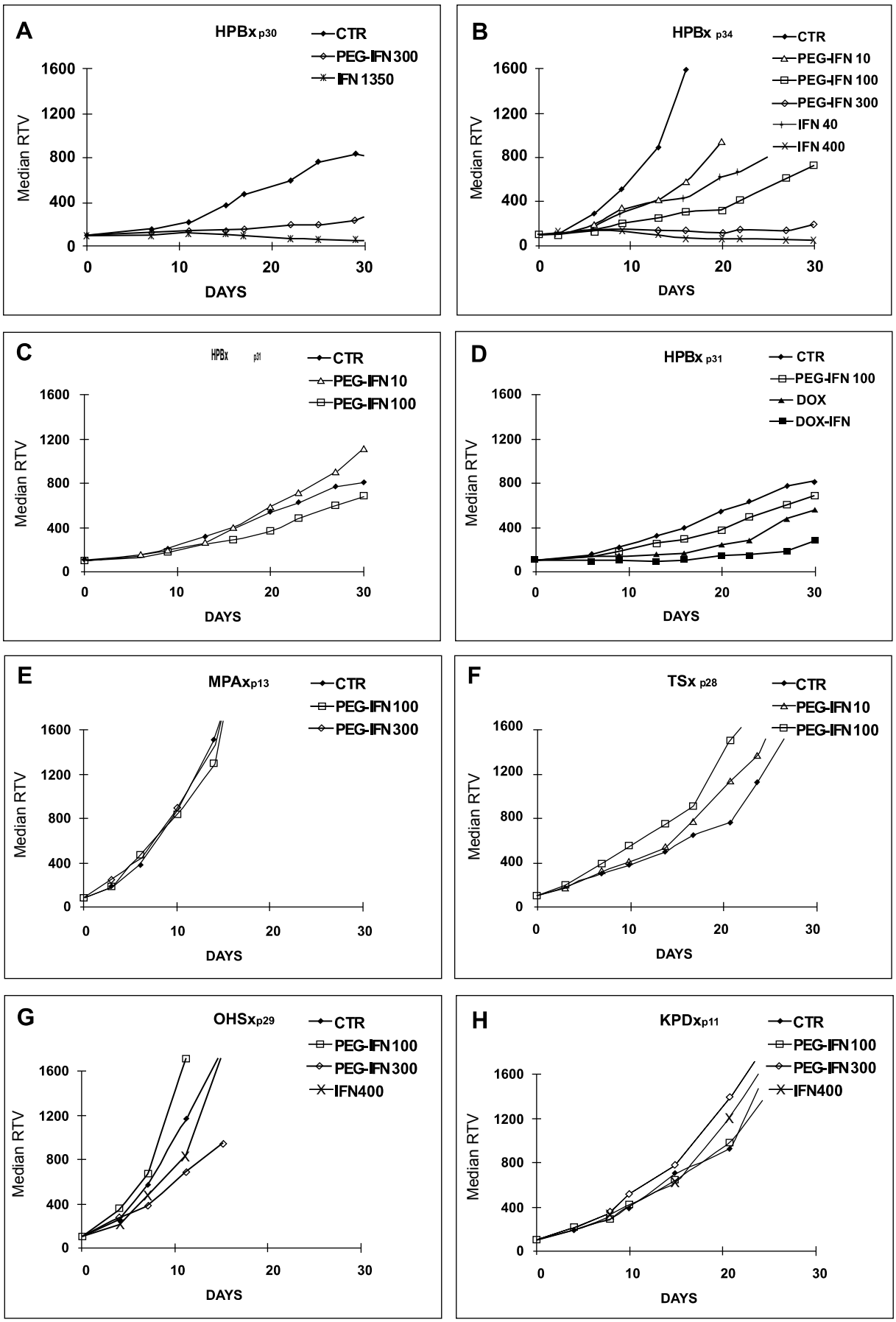
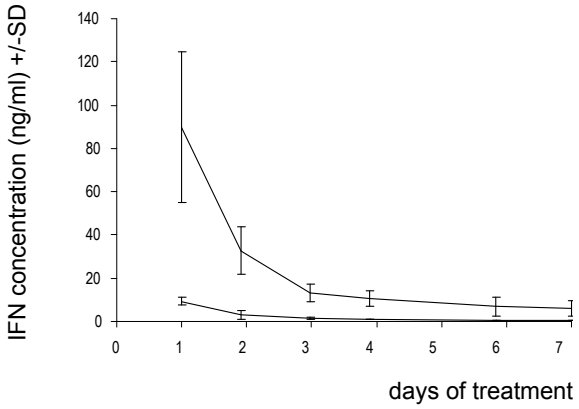


Figure 1: Growth response of osteosarcoma xenografts to treatment with PEGylated IFN (PEG-IFN), unconjugated IFN (IFN), doxorubicin (DOX) or a combination of doxorubicin and PEGylated IFN (DOX-IFN) compared to a control group (CTR). Doses are given in equivalents to g/kg/week. IFN was always given daily. PEG-IFN was given weekly in Figure A, C, D, E and F and every third day in Figure B, G, H. Doxorubicin was given at the maximal tolerated dose of 8 mg/kg 24 hours after start of treatment with PEG-IFN 100 (DOX-IFN). Volume is annotated as median relative tumour volume (RTV).



dose/day	1	2	3	4	6	7
10	9.3 +/- 1.8	3.1 +/- 2	1.5 +/- 0.7	1.05 +/- 0.1	0.62 +/- 0.1	0.59 +/- 0.2
100	89.8 +/- 35	32.7 +/- 11	13.2 +/- 4	10.8 +/- 3.5	7.1 +/- 4.3	6.0 +/- 3.7

Figure 2: Elimination half-life of PEGylated IFN in mouse serum. Serum concentration of radiolabeled PEGylated INF-α2b in NCR mice following a single treatment with a dose of 10 and 100 g/kg.

with the three xenografts HPBx, TSx and MPAx, representing the one sensitive and two resistant xenografts, respectively.

Global changes in gene expression: Treatment with PEGylated IFN caused a significant change in the expression detected by 300 probes in HPBx, 325 in TSx and 1,777 in MPAx when comparing the treated and untreated xenografts pair-wise. The probe lists were filtered to identify probes that were specific for the sensitive and resistant xenografts (see Supplementary Tables 1-3) and were further classified according to biological and functional characteristics (Supplementary Table 4).

Genes affected by IFN in all xenografts: Ninety-nine of the probes detecting significant expression changes upon IFN treatment were common for all three xenografts. Five of these probes showed opposite effects in some of the three xenografts and were removed. This resulted in a set of 94 probes, representing 79 genes; 75 genes were up-regulated and only four were down-regulated in all xenografts. Mean fold change for these genes were 2.4, 6.6 and 3.9 in HPBx, TSx and MPAx, respectively (Supplementary Table 1). This group contains several core members of the IFNα signalling pathway (*STAT1, ISGF3G, IRF7*) and central well characterized ISGs (*OAS1-3, MX1, EIF2AK2*, members of the *HLA* and *PARP* families, and *ISG15*) (Supplementary Table 1). As expected, these genes were enriched in immunity related gene ontology groups and pathways (Supplementary Table 4A).

Sixty-six of the 79 common genes were identified as known ISGs in the Interferome database (www.interferome.org; Samarjiwa et al., 2008). Thirty-six of these 66 genes were found to contain putative ISRE and 16 had a putative STAT1 binding site in the promoter. The high number of ISRE-containing genes illustrates the induction of a specific IFN response in all three xenografts examined. For the majority of genes, this effect of IFN treatment was more pronounced in the resistant xenografts.

Genes differentially expressed only in the sensitive xenograft: One hundred and twenty-eight probes identified genes that were differentially expressed upon treatment with PEGylated IFN-α only in the sensitive xenograft HPBx. 72 of these probes, representing 70 unique genes, had a fold-change difference of ≥ 1.5 between the sensitive and each of the two resistant xenografts. 24 of these genes were induced and 48 were repressed. Only seven of these genes were identified as known ISGs by the Interferome database and only one of the seven had a putative ISRE in the promoter (Supplementary Table 2).

The 70 genes were significantly enriched for gene ontology annotations related to signalling (MF00016) and receptors (BP00108), extracellular matrix (MF00179) and actin binding protein (MF00262), development (BP00193) or cell adhesion (BP00124) and KEGG pathway calcium signalling pathway (hsa04020) (Supplementary Table 4B).

IFN down-regulated a number of genes related to the extracellular matrix of connective tissue and its maturation, including the collagens *COL2A1*, *COL9A1* and *COL10A1*, osteomodulin (*OMD*), matrilin 4 (*MATN4*), and metallopeptidase 3 (*MMP3*). The adhesion receptor galectin-7 (*LGALS7*) and *LOC728910* similar to galectin-7 is down-regulated whereas galectin-9 (*LGALS9*) and the highly similar *LGALS9C* are up-regulated.

Up-regulated genes related to developmental processes included chordin-like 2 (*CHRDL2*) involved in osteo- and myoblast differentiation and *T1560* involved in thyroid development. Down-regulated genes included the Wnt pathway inhibitor dickkopf1 (*DKK1*), chordin (*CHRD*) which is an inhibitor of bone morphogenetic proteins, SRY (sex determining region Y)-box 8 (*SOX8*) involved in pro-osteoblast differentiation, secretoglobin (*SCGB3A2*) involved in lung development, and the tyrosine kinase receptor ephrin-A1 (*EFNA1*) involved in development, tumourigenesis and metastasis.

Genes with similar IFN response in the two resistant xenografts: Of the probes detecting significant expression changes, 58 were common only to the two resistant xenografts TSx and MPAx, but not significantly changed in the sensitive HPBx. Only 19 of these probes, representing 17 unique genes, were similarly regulated in both resistant xenografts and had a fold-change above 1.5 when compared to the sensitive xenograft (Supplementary Table 3). Eight of these genes are known as

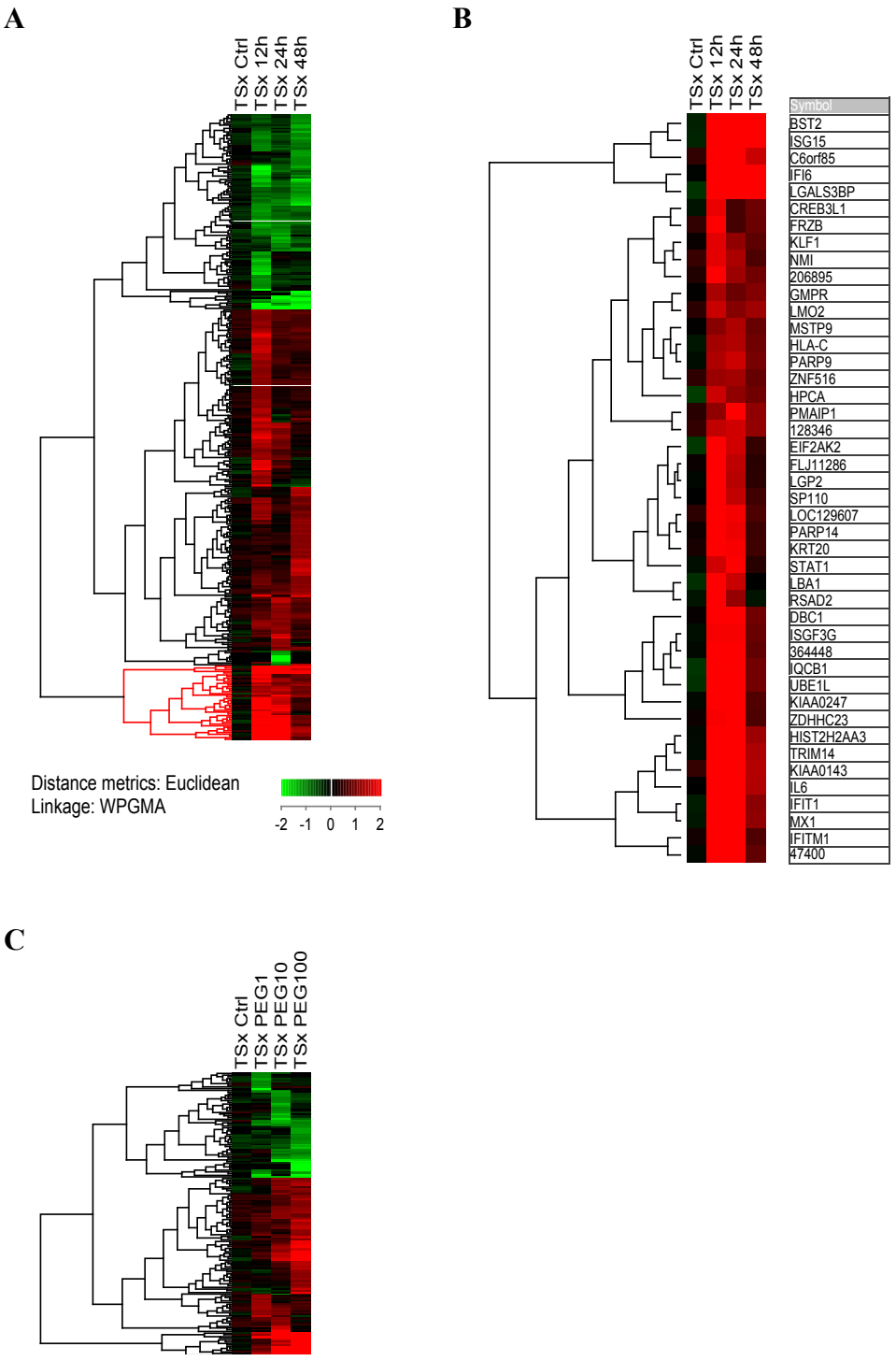


Figure 3: Hierarchical clustering of gene expression patterns in initial time and dose response experiments. A. 369 differentially expressed probes in xenograft TSx treated with 100 g/kg/week PEGylated IFN-α2b for 12, 24 and 48 hours. B. Expanded view of lower subcluster (red) from A, showing induction of numerous interferon-regulated genes C. 168 differentially expressed probes in xenograft TSx treated with 1, 10 or 100 g/kg/week PEGylated IFN-α2b for 24 hours. An untreated control sample (TSx Ctrl) was also included. The signal of each probe is given as log2 of the ratio between the treated and untreated samples that was co-hybridized to the arrays, represented as mean value of two replicated assays. The probes presented in the clusters were at least 2 times up- or down-regulated in at least 1 of the treated samples in the cluster."

ISGs in the Interferome database of which one had a putative ISRE and five a putative STAT1 promoter binding site. Functional annotation analysis yielded enrichment in the Panther ontology group MF00001: receptor.

Gene expression analysis of combination of doxorubicin and PEGylated IFN: Limma analyses revealed no significant differences in the gene expression patterns of the xenografts treated with IFN alone compared to the combination with doxorubicin at the time point investigated (data not shown).

Discussion

The effect of IFN on tumour growth

In an earlier series of 14 osteosarcoma xenografts in nude mice, natural buffy coat-derived IFN- α at a daily dose of 200,000 IU induced tumour regression or growth arrest in five, partial growth inhibition in eight whereas one tumour could only be

arrested at a higher dose (Brosjo, 1988). In contrast, in this report only one of five xenografts were sensitive to PEGylated IFN-α2b. This may be explained by differences in the tumour panels or the therapeutic agents. It may be relevant that three of our tumours (including the sensitive one) were exposed to chemotherapy before xenografting, whereas all the Karolinska tumours were chemotherapy naïve. The sensitive xenograft being the only one of metastatic origin is an observation of uncertain significance: small series with IFN in metastatic osteosarcoma did not indicate clinically relevant efficacy (Hans Strander and Stefano Ferrari, personal communications). Furthermore, our xenograft lines were maintained over a longer time before the experiments. Although passage number did not seem to affect response to chemotherapy in a previous study on this panel (Bruheim et al., 2004), we can not exclude that primary IFN resistance may be caused by passage-related genetic or epigenetic alterations over time. As human IFNs are not thought to evoke a host response in mice (Balkwill, 1985), it seems unlikely that a different immune status of our animals would explain the different treatment response.

The difference in response compared to previous reports may also be due to the type of IFNs used may also be due to the type of IFNs used. Natural IFN, as used in the Karolinska experiments, contains a mixture of α-interferon subtypes that have varying properties and anti-proliferative activity (Thomas and Balkwill, 1991; Foster et al., 1996; Foster and Finter, 1998; Yano et al., 2006). It is also possible that PEGylation may directly influence the antitumour effect. Natural α-IFNs have been shown to have higher anti-tumour activity in a small panel of osteosarcoma xenografts compared with recombinant IFN- α2c (Bauer et al., 1987), but have not been directly compared with PEGylated IFN-α. Unconjugated and PEGylated IFN-α2 a induced similar growth inhibition and expression profiles in melanoma-xenografts (Certa et al., 2003; Krepler et al., 2004), whereas growth inhibition in hepatocellular carcinoma xenografts was stronger for PEGylated IFN-α2b (Yano et al., 2006). Significant variation in the IFN-related response depending on the targeted cell line and IFN–α subtype has been reported by others (Balkwill et al., 1985; Foster and Finter, 1998; Yamaoka et al., 1999; Yanai et al., 2001). In our panel, xenografts resistant to PEGylated IFN were similarly resistant to unconjugated IFN. Complete growth arrest in the sensitive xenograft was only obtained by unconjugated IFN, but this could be due to the higher dose administered per week compared to the PEGylated moiety. Importantly, lack of effect in our model system on tumour growth is only reflecting the absence of a direct antitumour effect of IFN and does not rule out clinically important effects mediated through immune- or angiogenesis-mediated mechanisms.

Changes in gene expression in response to IFN

With only one sensitive xenograft, we limited the microarray analysis to compare the one sensitive to two resistant xenografts. Obviously such a limited analysis cannot provide definite answers, but may indicate a list of candidate genes and pathways related to IFN treatment response. We restricted the investigation of the transcriptional response to stimulation with PEGylated IFN, as previous studies have shown that unconjugated and PEGylated IFN give indistinguishable transcriptional patterns and are equally potent activators of IFN gene expression (Certa et al., 2003; Krepler et al., 2004).

When comparing the sensitive and the resistant osteosarcoma xenografts we identified 79 genes with similarly altered expression after IFN treatment regardless of tumour response. A subset of 36 genes contained ISRE, indicating a functional JAK/STAT signalling pathway in all three xenografts. Genes exclusively induced in the sensitive xenograft rarely contained ISRE; a comparable group of genes was observed earlier in an IFN sensitive melanoma cell line and termed IFN secondary response genes (Certa et al., 2003). It was postulated that resistance to IFN is caused by abnormal secondary signalling rather than by primary defects in induction of JAK-STAT signalling pathway (Certa et al., 2003; Holko and Williams, 2006).

LGALS9, and the similar LGALS9C, are among the ISGs induced by IFN in the sensitive xenograft. Galectins are evolutionarily highly conserved β-galactoside-binding lectins involved in basic cellular mechanisms (cell interaction, proliferation, migration, apoptosis, mRNA splicing) and modulation of immunity. LGALS9 expression is correlated with a better prognosis in malignant melanoma (Kageshita et al., 2002), and is possibly involved in IFN-induced apoptosis in the melanoma cell line WM9 (Leaman et al., 2003). Two other members of the galectin family, *LALS7* and *LOC728910*, were found to be down-regulated, and these proteins have previously been described both as a positive and negative regulatory factors in tumour development, involved in proapoptosis, neoangiogenesis and metastatic tendency through metastatic proteins such as MMP9 (Saussez and Kiss, 2006).

IFN-α has been shown to induce bone differentiation in a xenotransplanted human osteosarcoma (Forster et al., 1988). Osteosarcomas are known to express several bone morphogenetic proteins (BMPs) (Bauer and Urist, 1981; Gobbi et al., 2002; Khan et al., 2008), the central proteins in the fine-tuning of bone development**.** Both CHRD, a regulator of dorsoventral patterning in early embryogenesis, and CHRDL2, a structurally related protein expressed preferentially in chondrocytes of developing cartilage, are BMP binding inhibitors of bone differentiation (Zhang et al., 2007)**.** In our sensitive xenograft, IFN induced upregulation of *CHRDL2* whereas *CHRD* was down- regulated, the significance of which is unclear.

Further, the transcription factor *SOX8* was repressed as a response to IFN treatment. SOX8 is involved in early chondrogenesis and SOX8-deficient mice display an osteopenic phenotype. SOX8-deficient preosteoblasts proliferate slowly probably caused by RUNX2-induced exit from the cell cycle (Schmidt et al., 2005), and the reduced expression of *SOX8* may thus be related to the tumour response.

Finally, *DKK1* was down-regulated following IFN treatment in the sensitive xenograft only. DKK1 inhibits Wnt signalling and impairs osteoblast function. DKK1 has been shown to be expressed maximally at the periphery of the tumour and it is thought that DKK1 can contribute to tumour expansion by inhibiting repair of the surrounding bone (Lee et al., 2007). Osteosarcoma patients have elevated DKK1 serum levels and the surviving fraction of circulating osteosarcoma cells is proportional to DKK1 levels in a xenograft model (Lee et al.,

2007); it has been suggested that DKK1 levels could be used to monitor the effect of osteosarcoma treatment, and this might be applicable also to interferon. However, our findings contrast *in vitro* data of interferon induced overexpression of DKK1 in a hepatoblastoma cell line (Qu et al., 2007).

In summary, several genes involved in cell adhesion and osteogenic tissue development were altered by IFN in the sensitive xenograft. However, the analysis of only one sensitive xenograft, and the fact that the magnitude of change in these genes appeared low, makes caution in the interpretation imperative.

Our observations that combined treatment with IFN and doxorubicin has an increased efficacy indicate that IFN may moderate response to other cytotoxic drugs. An exploratory analysis did not find transcriptional changes of combined IFN and doxorubicin as compared to IFN alone. Doxorubicin has been shown to induce both cell cycle regulators and apoptotic genes in hepatocellular cancer cell lines (Wang et al., 2009). The lack of specific mRNA changes in this report may point at a posttranscriptional regulation by the combination in the sensitive xenograft. Others have shown that one of the many genes induced by IFN is the tumour suppressor TP53 (Takaoka et al., 2003), and that IFN-related genes such as ISGF3G directly influence p53 expression (Munoz-Fontela et al., 2008). Doxorubicin can induce apoptosis through p53 in osteosarcoma cells (Yuan et al., 2007; Yuan et al., 2008). In this study, no change in TP53 mRNA levels was observed between IFN treated and untreated osteosarcoma xenografts, but we cannot rule out effects at the protein level. Finally, it has been shown that IFN-α can mobilise quiescent leukemia stem cells, thus making them sensitive to chemotherapy (Essers et al., 2009). It remains to be seen if such mechanisms also can be exploited in non-haematological cancers.

Conclusions

We found a direct antitumour effect of IFN in one of five xenografts. At the doses tested, both types of IFN reduced the growth rate, but only unconjugated IFN induced growth arrest. Known ISGs were induced independent of tumour response. The antitumour response seen in one xenograft may be related to interference with genes involved in cell adhesion or osteogenic tissue development. Our preliminary data of combined treatment with IFN and doxorubicin are encouraging and should be further explored.

Competing interests

The authors declare that they have no competing interests.

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