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Distinct Mechanisms of Interferon-Gamma and Tumor Necrosis Factor-Alpha Action in Oncogene-Transformed Mouse Fibroblasts

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The potential mechanisms of interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha action on tumor cells have been investigated in a model of mouse fibroblasts transformed by distinct retroviral vectors carrying the v-mos, c-myc, and v-Ha-ras oncogene, respectively. Treatment with both cytokines not only caused growth inhibition of v-mos- and c-myc-transformants, but also a reversion of transformation-induced suppression of major histocompatibility complex (MHC) class I antigen expression in all transformed cell lines. The phenotypical reversion of transformants was preceded by a selective modulation of LTR-controlled oncogene expression. TNF-alpha primarily affected stability of oncogene-specific RNAs without influencing the activity of retroviral promoters. In contrast, IFN-gamma was effective at the transcriptional level, apparently due to inhibition of LTR activity as revealed from reduced CAT activity in IFN-gamma-treated LTR-CAT transformants. This IFN-gamma-mediated down-regulation of retroviral promoter activity seemed to be selective for Moloney-virus-derived promoters, since the activity of other viral and cellular promoters was not suppressed by IFN-gamma.

Key words: cytokines, gene expression, retroviral promoters, antitumoral activities, phenotypical reversion

Interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha are cytokines, which exhibit multiple biological activities including antiviral, antitumoral, and immunomodulatory action [1–8]. In sensitive tumor cells, cytokine treatment causes profound changes in the expression of genes involved in the regulation of cell proliferation, differentiation, and cell-surface antigen expression [3–7, 9–12]. To investigate whether the antineoplastic activities of IFN and TNF correlated with modulation of oncogene expression, fibroblasts transformed by defined LTR-controlled oncogenes of different transforming potential have been demonstrated to be a useful model [13–16]. In such transformants, murine recombinant IFN-gamma causes

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inhibition of LTR-controlled v-mos, c-myc, and v-Ha-ras oncogene expression due to reduction in gene transcription suggesting an effect of IFN-gamma on retroviral promoter activity [17]. However, the detailed molecular mechanisms of IFN-gamma action on LTR-activity remain largely to be defined. We here show that IFN-gamma-mediated inhibition of retroviral gene transcription is selective for promoters closely related to the Moloney sarcoma virus, since other viral and cellular promoters were not down-regulated by IFN-gamma treatment. In contrast, rTNF-alpha inhibits LTR-controlled oncogene expression at the post-transcriptional level without affecting the retroviral promoters.

MATERIALS AND METHODS

Cell Lines

The various oncogene-transformed NIH3T3 cell lines (3T3mos, 3T3myc, 3T3ras) as well as the respective control cell lines (3T3mos$^-$, 3T3SVX) were obtained by either transfection or retroviral infection with distinct retroviral vectors carrying the v-mos-, c-myc-, and v-Ha-ras-oncogene, respectively. All cell lines used have been recently described [17,18]. Cells were cultured in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum, 2.0 mM L-glutamine, antibiotics, and 400 μg/ml geneticin (G-418, Gibco, BRL). The in vivo and in vitro growth characteristics of retrovirus-transformed cells are summarized in Table I.

Cytokine Treatment

Both murine recombinant IFN-gamma (specific activity: 1.5×10^7 units/mg protein) and murine recombinant TNF-alpha (specific activity: 2.0×10^7 units/mg protein), produced in *Escherichia coli* and purified to homogeneity by Genentech (San Francisco, CA), were kindly provided by Boehringer Ingelheim, (Vienna, Austria).

Parental NIH3T3 cells and the different retroviral transformants were treated in monolayer cultures for the indicated lengths of time with 20 ng/ml recombinant (r)

TABLE I. In Vitro and In Vivo Growth Characteristics of Retrovirus-Transformed Mouse Fibroblasts

Cell lines	G-418 resistance (400 μg/ml)	Relative proliferation rate[a]	Growth in 1% serum[b]	Growth in soft agar (cloning efficiency in %)	MHC class I antigen expression[c]	Tumorigenicity[d]
3T3	−	1.0	−	0.1	+++	−
3T3mos$^-$	+	1.1	−	0.1	+++	−
3T3mos$^+$	+	3.6	+	90	+	+
3T3SVX	+	1.1	−	0.1	+++	−
3T3myc	+	3.7	+	10	(+)	+
3T3ras	+	4.3	+	90	(+)	+

[a]The in vitro proliferation rate of retrovirus-transformed cells was determined by liquid culture containing 10% fetal calf serum by ^{3}H-thymidine incorporation and compared to that of parental NIH3T3 cells.
[b]+ means a 4- (3T3myc) to 10-fold (3T3mos, 3T3ras) increase in cell no. after 6 days of incubation in MEM supplemented with low serum (1%) concentration.
[c]MHC class I antigen expression was determined by quantitative flowcytometry immunofluorescence analysis on Epics C using H-2^d-specific antibodies (see also Table II).
[d]Personal communication (3T3mos$^-$, 3T3mos$^+$: W. Ostertag, Heinrich-Pette-Institut, Hamburg, FRG; 3T3SVX, 3T3myc, 3T3ras: R. Schäfer, Ludwig Institute for Cancer Research, Bern, Switzerland).

rIFN-gamma and 10 ng/ml rTNF-alpha, respectively. Both cytokine concentrations did not cause cytotoxic effects on untransformed and transformed NIH3T3 fibroblasts.

Cytofluometric Analysis of MHC Class I Antigen Expression

MHC class I antigen expression of untreated and cytokine-treated cells (48 h) was determined by indirect immunofluorescence with the monoclonal antibodies 31-3-45 (H-2K^d) and 34-4-20S (H-2D^d) obtained from ATCC (Rockville, MD), using fluorescein isothiocyanate (FITC)-conjugated goat-antimouse Ig-antibodies (Medac, Hamburg, FRG) as a second reagent. The percentage of cells expressing H-2^d antigens was calculated upon subtraction of the respective background fluorescence determined for each group with the isotype-matched control antibody L243 (anti-HLA-DR) [19].

Promoter CAT Plasmids and Analysis of Promoter Activity

The recombinant CAT plasmids carrying either cellular or viral promoters were kindly provided by Dr. F. Wu (heat shock promoter; Hsp cat), Dr. P. Gruß (thymidine kinase promoter; Tk cat), [Dr. P. Kourilsky (H-2 promoter; H-2 cat), Dr. H.-J. Hauser (Rous sarcoma virus promoter; RSV cat), Dr. M. Grez (myeloproliferative sarcoma virus promoter (MPSV-cat), Moloney murine leukemia virus promoter (MoMuLV cat), Dr. U. Koszinowsky (murine cytomegalovirus promoter (MCMV-cat)]. Using a DNA-calcium phosphate precipitate [20,21], promoter CAT expressing cell lines were generated by cotransfecting 5×10^5 NIH3T3 cells with 50 μg of the appropriate promoter CAT plasmid and 5 μg pAG60 DNA. Stable transformants were isolated by selection in MEM supplemented with 400 μg/ml G-418. Extracts from both untreated and IFN-gamma (10-100 ng/ml, 48 h) as well as TNF-alpha (10-50 ng/ml, 48 h) treated transfectants were prepared, normalized for total protein content, and incubated with ^{14}C-labelled chloramphenicol for 4 h to determine CAT activity. The acetylated and nonacetylated reaction products were separated by thin layer chromatography as described [22]. Total radioactivity as well as radioactivity associated with the acetylated forms of chloramphenicol was counted by liquid scintillation and percent specific conversion was calculated according to the formula: percent specific conversion = (cpm of acetylated chloramphenicol/total cpm) $\times$ 100.

RESULTS

Cytokine-Mediated Inhibition of the Neoplastic Phenotype of Oncogene-Transformed Fibroblasts

IFN-gamma and TNF-alpha treatment of oncogene-transformed fibroblasts caused profound phenotypical changes as revealed by lack of focus formation and failure to form colonies in soft agar [16–18]. This was confirmed by analysis of in vitro growth rate in monolayer culture of v-mos-, c-myc-, and v-Ha-ras-transformants. As shown in Figure 1, both IFN-gamma and TNF-alpha affected the proliferative capacity of some of the oncogene-transformed cells. Compared to untreated transformants, IFN-gamma and TNF-alpha strongly reduced the growth rate of 3T3mos4 and 3T3myc2 cells during a 6-day continuous cytokine treatment (Fig. 1). In contrast, no significant inhibition of the proliferative capacity of v-Ha-ras-transformants was obtained using cytokine concentrations up to 50 ng/ml. Combined application of TNF-alpha and IFN-gamma caused complete cytostasis of v-mos and

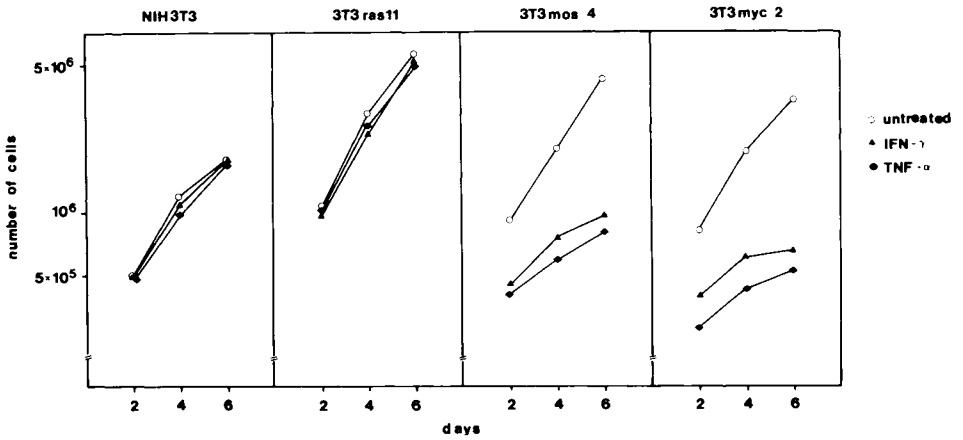

Fig. 1. Growth rate of oncogene-transformed mouse fibroblasts in the absence or presence of cytokines. Cells were seeded at low density (2.5×10^5/75 cm^2 flask/time point) to ensure exponential growth conditions for the time period analyzed. At the onset and every second day of culture, cells were either supplemented with fresh culture medium containing 20 ng/ml IFN-gamma or 10 ng/ml TNF-alpha. At the indicated time point, the total cell number was determined by microscopic counting. Results are expressed as mean of duplicate cultures: (○—○) untreated cells, (▲—▲) IFN-gamma-treated cells, (◆—◆) TNF-alpha-treated cells.

TABLE II. Cytokine-Mediated Modulation of MHC Class I Antigen Expression in Retrovirus-Transformed Cells

	Relative fluorescence intensity in % of untreated NIH3T3[a]			
Cell lines	Untreated	IFN-gamma (20 ng/ml)	TNF-alpha (10 ng/ml)	IFN-gamma/TNF-alpha (20 ng/ml/10 ng/ml)
NIH3T3	100	222	112	356
3T3mos$^-$	93	205	111	284
3T3mos$^+$	35	156	100	298
3T3SVX	98	201	106	344
3T3myc	12	103	62	192
3T3ras	18	143	122	263

[a]Background fluorescence with control antibody L243 range from 10–30 for all groups listed above and has been subtracted. $H2D^d$ specific fluorescence intensity of NIH3T3 was 121 (100%) on a log scale from 1–1,024.

c-myc transformants and a partial reduction in the proliferative capacity of v-Ha-ras cells (data not shown). Further, under the conditions of high serum concentrations (10% FCS) used in these experiments, the cytokines had neither an inhibitory nor a stimulating effect on the growth of parental NIH3T3 cells (Fig. 1) and the respective oncogene negative control cell lines (data not shown).

In some tumor models, absence of major histocompatibility complex (MHC) class I molecules is associated with increased tumorigenicity and metastatic capacity [23–25], suggesting that specific immune responses may play an important role in control of tumor growth. In accordance with these findings, we here show that oncogene-induced transformation of NIH3T3 cells causes inhibition of class I MHC antigen expression. V-mos-, c-myc-, and v-Ha-ras-transformants exhibit a significantly lower $H\text{-}2K^d$ (data not shown) and $H\text{-}2D^d$ (Table II) membrane expression as compared to parental NIH3T3 cells and control cell lines. However, treatment with either IFN-gamma or TNF-alpha enhanced the levels of membrane H-2K and H-2D

antigens on 3T3mos, 3T3myc, and 3T3ras cells. A typical experiment is showing in Table II, indicating the amount of H-2 antigens on cytokine-treated, oncogene-transformed cells was comparable to that of normal, nontransformed cells. Moreover, similar to results obtained with human tumor cells [26,28], a combined application of IFN-gamma and TNF-alpha caused a further enhancement of H-2 membrane expression in both nontransformed and transformed 3T3 cells (Table II).

Differential Mechanisms of Cytokine Action

Our recent data indicated that both IFN-gamma and TNF-alpha can interfere with the growth of oncogene-transformed cells due to a down-regulation of LTR-controlled oncogenes [17,18]. TNF-alpha-mediated inhibition of v-mos- and c-myc oncogenes was due to a selective mRNA degradation of these oncogenes as revealed from a reduced RNA half-life, but unchanged gene transcription [18]. In contrast, IFN-gamma suppressed the expression of LTR-controlled oncogenes v-mos, c-myc, and v-Ha-ras at the transcriptional level, apparently due to a direct effect on the activity of retroviral promoters [17]. To define further the molecular mechanism(s) involved and to investigate the selectivity of cytokine action on retroviral promoter activity, other viral promoters (SV, MCMV, RSV) as well as three distinct cellular promoters (Tk, Hsp, H-2) linked to the CAT gene were compared with MPSV/MuLV cat constructs for sensitivity to IFN-gamma and TNF-alpha in the respective transformants. As shown in Table III, IFN-gamma, but not TNF-alpha, treatment of stable MPSV and Mo-MuLV cat transformants reduced the activity of both MPSV-LTR and Mo-MuLV-LTR, as revealed from a 50% suppression of CAT activity. In contrast, both cytokines did not inhibit CAT-activity in six distinct promoter CAT-transformants analyzed (Table III). Rather, IFN-gamma treatment produced a slight enhancement of CAT-activity in the transformants expressing the CAT gene under control of the mouse class I MHC gene (H-2) promoter. The data suggest that IFN-gamma-mediated inhibition of promoter activity is probably restricted to certain retroviral LTRs closely related to Moloney virus.

TABLE III. Influence of Cytokine on CAT-Activity in Promoter CAT-Transformed NIH3T3 Cells

Promoter cat constructs		% specific conversion of ^{14}C-chloramphenicol[a]		
Promoter	Origin	Untreated	IFN-gamma[b]	TNF-alpha[b]
Hsp cat		95[c]	94[c]	96[c]
Tk cat	Cellular	97	95	96
H-2 cat		74	91	76
SV cat	DNA virus	52	50	55
MCMV cat		82	86	85
MuLV cat		99	47	95
MPSV cat	Retrovirus	96	51	90
RSV cat		49	47	51

[a]The CAT-dependent conversion of ^{14}C-chloramphenicol into monoacetylated 1 and 3 acetylchloramphenicol, respectively, was determined as described in Materials and Methods.
[b]Stable CAT-transformants were treated for 48 h with 100 ng/ml of IFN-gamma and 50 ng/ml of TNF-alpha, respectively.
[c]Percent specific conversion after induction (heat shock, 1 h, 42°C).

DISCUSSION

Our previous studies have demonstrated that IFN-gamma and TNF-alpha treatment of v-mos- and c-myc-transformed cells resulted in the loss of transformed properties. So far, the major changes observed in both IFN-gamma- or TNF-alpha-treated, transformed cells relate to in vitro growth characteristics in liquid culture and semisolid medium as revealed from strong inhibition of the proliferative capacity, lack of focus formation, and failure to grow anchorage-independent [16–18]. We here show that cytokine treatment of transformed cells caused significant phenotypical changes independent of the effect on growth characteristics. Thus, in comparison to oncogene-negative controls, in all three independently derived, transformed cell lines, H-2 expression was strongly reduced, independent of the type of oncogene used for transformation. Treatment with IFN-gamma and TNF-alpha, respectively, increased H-2 antigen expression to normal levels not only in v-mos- and c-myc-transformants, but also in v-Ha-ras-transformants (Table II), which are insensitive to both IFN-gamma and TNF-alpha treatment with regard to modulation of in vitro growth characteristics (Fig. 1) [17,18]. Furthermore, an enhanced H-2 expression upon combined treatment with IFN-gamma and TNF-alpha was observed in all cell lines tested (Table II). This finding is in accordance with recent reports showing that IFN-gamma and TNF-alpha synergistically upregulates both class I and class II MHC gene expression in various human cells [26–28].

Down-regulation of MHC antigens induced by selective oncogenic transformation provides a model to approach experimentally the question of which mechanisms cause suppression of MHC antigens on the cell surface of certain malignant tumors [29,30]. We have now obtained evidence for both transcriptional and post-transcriptional control of H-2 gene expression in transformed 3T3 cells (Seliger, manuscript in preparation). As there exists an inverse relationship between major histocompatibility complex antigen expression and tumorigeneity of some animal tumors [31,32], a suppression of MHC antigens might represent a possible mechanism for tumor cell escape from immunosurveillance. Accordingly, reversion to a normal MHC phenotype by IFN- and/or TNF-alpha treatment may be not only a marker of tumor cell response to cytokine treatment, but may, indeed, contribute to control of tumor growth via MHC antigen-dependent induction of host immune responses.

In regard to the molecular mechanisms of IFN-gamma action on oncogene expression in transformed cells, our recent studies indicated that IFN-gamma regulates expression of the Moloney LTR-controlled oncogenes by affecting the transcriptional activity of these retroviral promoters [17]. We have here addressed the question of selectivity of IFN-gamma action, using plasmid constructs, in which various cellular as well as viral promoters were linked to the marker gene CAT. The data show that IFN-gamma-mediated suppression of promoter activity appears restricted to Mo-MuLV-related LTRs, as no influence was noted on two DNA virus (MCMV, SV 40) and one other retrovirus (RSV) promoter (Table III). Moreover, the cellular promoters investigated remained unaffected (Hsp, Tk) or were even enhanced (H-2) (Table III). This was expected from earlier data indicating that IFN-gamma selectively induces a number of cellular genes including MHC genes [2,4,19,31,33]. It is commonly accepted that promoters/enhancers are regions where site-specific DNA-binding proteins interact, thereby controlling transcription [34–36]. In order to define the detailed mechanisms of IFN-gamma action on Mo-MuLV-related LTR promoters,

the identification of specific positive and negative regulated domains and the characterization of cellular proteins that specifically interact with these regions will be required.

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