Publications

I. Szalay, K., Zs. Rázga, E. Duda: TNF inhibits myogenesis and downregulates expression of myogenic regulatory factors myoD and myogenin. Eur. J. Cell Biol. (74) 391-398 (1997).

II. Szalay, K., Á. Domonkos, J. Kovács, T. Mikó, M. Sass, M. Rassoulzadegan, F. Cuzin, E. Duda: 45T-1, An established cell line with characteristics of Sertoli cells, forms organized aggregates *in vitro* after exposure to tumor necrosis factor alpha. Eur. J. Cell Biol. (78) 331-338 (1999).

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IV. Szalay, K., Á. Domonkos, A. Magyar, S. Beniczky, E. Madarász, E. Duda: The effect of TNF on the differentiation of non-hematopoietic cells. Cell Biol. Intl. (20) 225 (1996).

V. Domonkos, Á., **K. Szalay**, E. Galiba, Zs. Győrfy, J. Kovács, M. Sass, T. Mikó, E. Duda, M. Rassoulzadegan, F. Cuzin: Developmental changes in embryonic Sertoli cell cultures after exposure to tumor necrosis factor. Cell Biol. Intl. (20) 221 (1996).

VI. Duda, E., Á. Domonkos, **K. Szalay**, E. Galiba, J. Kovács, M. Sass, T. Mikó, M. Rassoulzadegan, F. Cuzin: Differentiation of Sertoli cell cultures after exposure to tumor necrosis factor. Eur. Cytokine Netw. (7) 164 (1996).

VII. Nagy, T., **K. Szalay**, Á. Domonkos, E. Galiba, E. Duda: Cytokine gene expressing tumor cell grafts increase the defensive capability of the host immune system. Cell Biol. Intl. (20) 230 (1996).

Annex

Photocopies of full papers

I.

TNF inhibits myogenesis and downregulates the expression of myogenic regulatory factors myoD and myogenin

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Myoblast – myogenic differentiation – tumor necrosis factor (TNF) – myoD – myogenin

The presence of TNF and other inflammatory cytokines and their receptors is detected during embryonic development, but our knowledge about the role of these proteins in differentiation and development is very limited. TNF modulates the synthesis and activity of a number of transcriptional proteins that regulate the activity of tissue specific genes, therefore it may play a role in normal development. Since its synthesis is upregulated by stress and infections, it may also participate in the induction of pathological developmental processes and malformation. We investigated the effect of TNF in an in vitro differentiation system using C2 myoblasts. This inflammatory cytokine exerted a positive effect on the early steps of the process: it enhanced the proliferation and aggregation of myoblast cells. In contrast, TNF strongly inhibited the expression of those myogenic transcription factors (myoD and myogenin), which are known to be responsible for upregulated activity of muscle specific genes (like the genes of the myofilament proteins), and blocked the synthesis of mRNAs of myogenic differentiation markers (like skeletal α-actin, myosin heavy and light chains). As a result, these cells did not synthesize myofilament proteins and the organization of myofilaments did not take place in TNF-treated myoblasts.

Abbreviations: DMEM Dulbecco's modified Eagle medium. – EGF Epidermal growth factor. – FGF Fibroblast growth factor. – MRF Myogenic regulatory factor. – PBS Phosphate-buffered salt solution. – RVC Ribonucleoside vanadyl complex. – TGF Transforming growth factor. – TNF Tumor necrosis factor α.

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Introduction

TNF is a pluripotent cytokine produced mostly by activated macrophages, but a number of other cell types can also synthesize it. The best known function of TNF is the coordination of the defensive forces of the immune system against invading pathogens. It plays a key role in killing cells infected by viruses, intracellular parasites or cells undergoing malignant transformation.

The presence of TNF and its membrane-bound precursor form was detected during fetal and neonatal development of mice [34], both in embryos [24] and in culture fluids during in vitro fertilization [33]. Mouse blastocysts express the 55 kDa receptor of TNF [26] and both receptor types have been identified in mouse embryos [13, 17]. The importance of TNF activity during development was indicated by experiments where repeated injections of neutralizing antibodies to TNF into pregnant mice resulted in growth retardation of the fetus [19]. The normal development of TNF or TNF receptor null mutant mice does not contradict this observation, as the activity of other members of the TNF family of proteins might provide sufficient backup for the lost activity.

TNF's role during embryonic development could parallel its roles in the immune system and during inflammation: it can influence the proliferation and differentiation of cells (as a cell type-specific growth and differentiation factor), determine survival or elimination of cells (inducing programmed cell death through its 55 kDa receptor) and can participate in the remodelling of tissues (as an inducer of both collagenases and extracellular matrix proteins). Although TNF influences the synthesis and/or activity of a number of important ubiquitous and tissue-specific transcription factors, which control developmental changes in different tissues, its role in embryonic development and differentiation is mostly unknown.

Proper development of a multicellular organism is extremely complex, the realization of the program requires precise spatial and temporal control of cell proliferation and coordinate expression of a large number of different genes.

Relatively simple in vitro differentiation systems were established, which can help us to study fragments of the complex problem of development.

The culture of C2 myoblast cells, where proliferating myoblasts fuse into myotubes is a well characterized, in vitro differentiation system. In this system morphological changes are accompanied by well characterized changes in the regulation of a number of tissue specific genes, like members of the actin and myosin gene families [36].

C2 cultures (and some other myogenic cell lines and primary cultures of myoblasts from muscle tissue) undergo many of the steps characteristic of terminal myogenic differentiation in vivo. Formation of multinucleated myotubes proceeds by cell aggregation and union of their plasma membranes. Cell fusion and the formation of myotubes lead to the accumulation of muscle-specific transcripts and corresponding proteins [7].

Myoblast differentiation is dependent on the expression of certain myogenic differentiation genes and affected by cell interactions with the extracellular matrix [12, 20, 27]. The myogenic regulatory factor (MRF) genes are expressed only in skeletal muscles. Induction of the expression of these genes both in vivo and in vitro initiates the skeletal muscle-specific differentiation program in a wide range of cell types [8, 31]. Members of the MyoD gene family of proteins act in concert with a variety of other ubiquitous (e.g. SP1, CTF, SRF) and tissue-specific (e.g. MEF-2) transcription factors to regulate myogenic promoters [25].

In C2 cells MyoD mRNA is found both before and after differentiation. The reason why MyoD fails to activate myogenic genes in growing myoblasts is, that active cyclin D1-Cdk 4 complexes, that drive the cell cycle, inhibit MyoD activity [14]. Commitment to myogenesis is facilitated by autoactivation of the MyoD promoter by MyoD protein [30]. Myogenin (Myf4 codes for the human homologue) is expressed in every known muscle cell line and therefore may be the most critical factor for the maintenance of the differentiated phenotype [2]. Serum components, basic FGF, TGF-β and EGF, agents that suppress differentiation of muscle cells in vitro, downregulate the activity of the Myf4 gene [4, 15]. In proliferating myoblasts, myogenin fails to be expressed and its synthesis starts only at the onset of differentiation [3, 35].

Materials and methods

TNF and antibodies

The human TNF α gene was isolated from a human DNA library in our laboratory (Tóth, M., 1985, unpublished). Mutants coding for TNF molecules partially resistant to processing were produced and colonies were selected which produced more membrane-bound than soluble TNF.

Recombinant human TNF α was purified to homogeneity from E. coli cells overproducing the cytokine (Mai et al., 1987, unpublished). It had a specific activity of >20 U per ng protein and <0.05 ng endotoxin per mg protein. Its purity – based on silver stained SDS gel electrophoretic analyses – was over 95 percent.

Polyclonal rabbit and monoclonal mouse anti-TNF antibodies were produced and affinity purified in our laboratory, according to conventional methods.

Cell culture

C2 mouse myoblast cell line [36] clone 7 was a generous gift of Prof. P. Amati (Univ. di Roma La Sapienza, Rome/Italy). Cells were maintained as undifferentiated myoblast in Dulbecco's modified Eagle's medium (DMEM) purchased from SERVA (Heidelberg/Germany) supplemented with 10% fetal calf serum (Inst. Jacques Boy, Reims/France), 100 U/ml penicillin G and 100 μg/ml streptomycin, at 37 °C. Cell cultures were split every 2 or 3 days.

M9 cells are genetically modified HeLa cells, produced in our laboratory. Transformed with a DNA construct coding for a slightly modified human TNF protein, they overproduce the transmembrane form of TNF. The mutant TNF is more resistant to proteolytic processing than the wild-type cytokine and accumulates in the plasma membrane of the producing cells. High-level expression of TNF is controlled by SV40 and TNF promoter sequences in this construct. The cells are grown in DMEM with 1 to 5% serum.

Preparation and staining of sections of cell aggregates

C2/7 myoblast cells were grown in 24-well tissue culture plates. Cell monolayers and aggregates were fixed in 4% formaldehyde solution for 1 h at 4 °C. The fixed cultures were embedded in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN/USA) and frozen by petroleum ether cooled on a liquid nitrogen bath. Sections were cut at 15–20 μm in a cryostat, mounted on 0.4% gelatine-coated glass slides and dried overnight at 4 °C. Sections were stained using chresyl violet or hematoxylin-eosin technique.

Electron microscopy

For electron microscopy cell monolayers and aggregates were fixed in phosphate-buffered 3% glutaraldehyde, postfixed in phosphate-buffered 1% osmium-tetroxide, dehydrated in graded series of ethanol, washed in propylene oxide, and finally embedded in TAAB812 (TAAB). Thin sections were stained with uranyl acetate and lead citrate. Sections were investigated with a Philips CM10 electron microscope at 60 kV accelerating voltage.

[^{3}H]Thymidine incorporation assay

The proliferation of myoblasts in the presence of TNF was monitored by [^{3}H]thymidine incorporation. Cells were plated at a density of 10^5 cells/ml/well, using 24-well plastic culture dishes. Cells were allowed to attach in DMEM with or without TNF (1000 U/ml). Fresh medium was added on days 3 and 6 of culture, and experiments were terminated on day 10. Cells were labeled with 10 μCi/ml [^{3}H]thymidine (25 Ci/mmol, Amersham) in a volume of 0.25 ml/well of culture medium for 2 h at 37 °C. Then medium was removed, cells were washed and harvested in 0.3 ml of 1% sodium laurylsulfate (SDS), disrupted by sonication (on ice), precipitated onto nitrocellulose filter discs by 10% trichloroacetic acid (TCA) and washed repeatedly with TCA and finally with 70% ethanol. The amount of [^{3}H]thymidine incorporated into DNA was determined by liquid scintillation counting in a toluene-based scintillation fluid.

Plasmids and probes

Plasmids and probes used in this study are listed in Tab. 1. *1.* The probe for the mouse fast myosin light chain ($MLC1_F$) was composed of a DNA fragment ranging from Hpa I to Bam HI and including all of exon 1 and a portion of intron 1 of the mouse *MLC_F/$MLC3_F$* gene cloned in pSP64: pMLC1$_F$ [10]. *2.* Plasmid MHC 2.2 was used as a probe for the embryonic mouse myosin heavy chain (MHCemb). The *MHCemb* sequence was cloned into pBR327 [32]. *3.* The probe for the mouse skeletal α-actin ($\alpha_{SK\text{-}actin}$) was isolated from a pBR322 derivative, pAM91-1 [22]. *4.* pEMCIIs was used as a probe for the mouse myogenic regulatory factor, MyoD. *MyoD* gene was inserted into pEMSV [11]. *5.* We used a human *myf4* sequence as a probe for myogenin, as this cDNA is highly homologous to the mouse myogenin gene. *Myf4* was cloned into Bluescript [2]. All these plasmids were kindly provided by Prof. P. Amati (Univ. di Roma La Sapienza, Rome/Italy).

Tab. I. Molecular probes used in these studies.

	Plasmid	Vector	Marker	E. coli strain	Restriction enzyme	Fragment size (bp)
MyoD	pEMCIIS	pEMSV	Ap	DH5α	Eco RI	2000
myf4	pMyf4	pBluescript	Ap	DH5α	Eco RI	1300
myosin heavy-chain (embryonic)	pMHC2.2	pBR327	Tc	JM109	Pst I	150 320
myosin light-chain (fast)	pMLC1$_F$	pSP64	Ap	DH5α	Hpa I-Bam HI	500
α-skeletal actin	pAM91-1	pBR322	Tc	JM109	Pst I	200

Ap Ampicillin. – Tc Tetracycline.

Transformation of E. coli

Cultures of E. coli (DH5α or JM109) were grown at 37 °C in YMG medium, up to optical density of 0.48 at 600 nm. Cells were chilled on ice for 10 min, centrifuged at 4000 rpm for 10 min at 4 °C, and the pellet was resuspended in half of the original culture volume of a solution containing 0.1 M $MgCl_2$ and 10 mM Tris-HCl, pH 8.0. After centrifugation at 4000 rpm for 10 min at 4 °C the pellet was resuspended in 1/25 of the original volume in a solution containing 0.1 M $CaCl_2$, 10 mM Tris-HCl, pH 8.0 and 10 % glycerol.

Up to 40 ng of plasmid DNA was incubated with 0.2 ml of competent E. coli cell mixture for 40 min on ice, then shocked in a water bath, preheated to 42 °C for 2 min. 0.8 ml of Luria-Bertani (LB) medium was added to the tube, and after 1 h of incubation at 37 °C in a shaker, cells were spread by the top agar procedure onto selective media containing 50 μg/ml ampicillin or 12.5 μg/ml tetracycline depending on the plasmid type, followed by incubation at 37 °C overnight.

Isolation of plasmid DNA

Bacterial cells were grown in 3 ml of LB medium containing 50 μg/ml ampicillin or 12.5 μg/ml tetracycline overnight at 37 °C. Cells were centrifuged at 13000 rpm for 2 min and the pellet was resuspended in 0.2 ml of a lysis buffer (50 mM Tris pH 8.0, 50 mM EDTA, 8 % sucrose, 5 % Triton X-100 and 4 mg/ml lysozyme (Reanal Fine Chemicals, Budapest/Hungary). After boiling for 1 min and centrifugation for 10 min at 13000 rpm, plasmid DNA was precipitated from the supernatant by 0.2 ml of cold isopropanol at room temperature. After centrifugation at 13000 rpm at 4 °C for 10 min, DNA was dissolved in distilled water.

Electroelution

Plasmids were digested with the appropriate restriction endonucleases. Each preparation was subsequently size fractionated on 1 % or 2 % agarose gels, depending on the fragment size. After running the gel the bands of interest were localized using a long-wavelength UV lamp. The slice of agarose containing the DNA fragment was cut out and placed in a TBE-filled dialysis bag. The bag was immersed in an electrophoresis tank and electric current (110 V) was passed through the bag. After 1 h electrophoresis the buffer surrounding the gel slice was recovered and the DNA was precipitated with 0.1 volume of sodium acetate (pH 5.2) and 2 volumes of 96 % ethanol, overnight at −20 °C. After centrifugation (13000 rpm, 15 min) the pellet was rinsed with 70 % ethanol, dried and resuspended in water.

For Northern blots the following fragments were isolated by this method: 500 base pair (bp) Hpa I-Bam HI fragment of the plasmid MLC1$_F$, 320 bp Pst I fragment of pMHC2.2, 200 bp Pst I fragment of pAM91-1, 2000 bp Eco RI fragment of pEMCIIs, 1300 bp Eco RI fragment of pMyf4 (see Tab. I).

Isolation of RNA

RNA samples were isolated from cultured animal cells by using the ribonucleoside vanadyl complexes (RVC) method. Cells ($5–10 \times 10^6$) were harvested from confluent cultures by trypsinization, centrifuged at 4000 rpm for 10 min at 4 °C in PBS. The pellet was resuspended in lysis buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 1 % NP-40) and 1/15 volume of 200 mM RVC (Sigma, St. Louis, MO/USA) was added. After centrifugation at 13000 rpm at 4 °C, the supernatant was mixed with equal volumes of extraction buffer (0.2 M Tris-HCl, 0.35 M NaCl, 20 mM EDTA, 1 % SDS, pH 8.0), a mixture of chloroform and isoamyl alcohol (49:1) and Tris-EDTA-saturated phenol. After brief shaking the mixture was separated by centrifugation at 13000 rpm at 4 °C, the aqueous phase was recovered and re-extracted with phenol/chloroform, centrifuged as above and re-extracted once with chloroform and centrifuged again. RNA was precipitated with 0.1 volume of 3 M Na-acetate (pH 5.2) and 2 volumes of ethanol at −20 °C overnight.

Northern blotting

Total RNA (20 μg/lane) was electrophoresed in a 1.25 % agarose/formaldehyde gel, and transferred to a nylon membrane (Hybond-N, Amersham) with 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). Filters were baked for 1 h at 80 °C. Integrity and relative amounts of RNA were checked by ethidium bromide staining of ribosomal RNA on parallel gels. The concentration and the purity of RNA were determined by spectrophotometry at 260 and 280 nm.

Prehybridization was performed in 0.25 M disodium phosphate, 7 % SDS (pH 7.2) and 250 μg/ml sonicated chicken blood DNA, at 65 °C for 1 h.

Hybridization was performed overnight in the same buffer containing 10^6 cpm/ml of [^{32}P]-cDNA probes, labeled by random primer synthesis with [^{32}P]dCTP (Izotóp Intézet Kft., Budapest/Hungary) using the Oligolabeling kit (Pharmacia, Uppsala/Sweden), and separated on Sephadex G-50 column. Blots were washed at 65 °C under high stringency conditions: two times for 30 min in 20 mM Na_2HPO_4, 5 % SDS, pH 7.2, and 1 or 2 times for 30 min in 20 mM Na_2HPO_4, 1 % SDS, pH 7.2. Following hybridization, filters were exposed for 1 to 10 days at −70 °C to X-ray films using intensifying screens. A glyceraldehyde-3-phosphate dehydrogenase (PGAD) probe (pHcGAP, from ATCC, Rockville, MD/USA) was used to determine the approximate RNA contents in the respective lanes.

Results

TNF induces the aggregation of C2/7 myoblast cells. To investigate the effect of TNF on myoblast differentiation we exposed subconfluent cultures of C2/7 myoblast cells to TNF. The cells were seeded in 24-well plates and exposed to 50 ng/ml human recombinant TNF. The additions of TNF caused a spectacular rearrangement of myoblast cells in the cultures. The cells assembled into clumps and formed extensive multicellular aggregates in a few days (Fig. 1). Using large culture

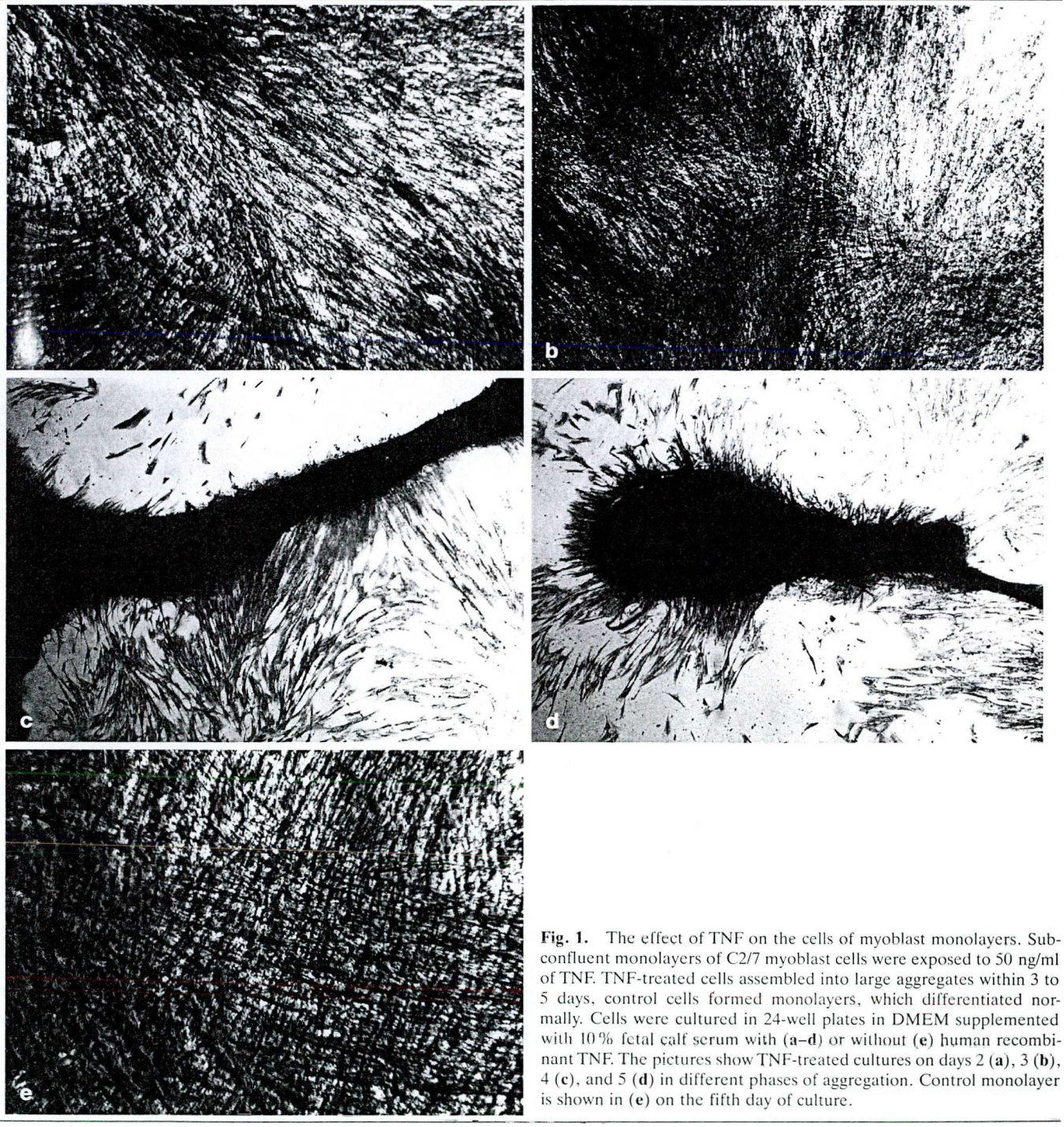

Fig. 1. The effect of TNF on the cells of myoblast monolayers. Subconfluent monolayers of C2/7 myoblast cells were exposed to 50 ng/ml of TNF. TNF-treated cells assembled into large aggregates within 3 to 5 days, control cells formed monolayers, which differentiated normally. Cells were cultured in 24-well plates in DMEM supplemented with 10% fetal calf serum with (**a–d**) or without (**e**) human recombinant TNF. The pictures show TNF-treated cultures on days 2 (**a**), 3 (**b**), 4 (**c**), and 5 (**d**) in different phases of aggregation. Control monolayer is shown in (**e**) on the fifth day of culture.

plates and appropriately higher number of cells aggregates of several mm size were easily produced.

The accumulation of myoblast cells was dependent on TNF and addition of polyclonal anti-TNF serum completely blocked the movement of the cells. Control cultures of myoblasts remained in ordered monolayers for prolonged times and did not show any tendency for aggregation during the first five days of culture (Fig. 1). As TNF has a transmembrane form, which could elicit only juxtacrine effects (more amenable for inducing spatially restricted processes), we used M9 cells, producing the transmembrane form of human TNF, to investigate whether the membrane-bound form of TNF can also induce the mobilization and aggregation of myoblasts. M9 cells are genetically modified HeLa cells, transformed with a DNA construct coding for a processing-resistant human TNF protein. In the transforming DNA construct high-level expres-

sion of TNF is controlled by a combination of transcription elements derived from SV40 and TNF promoter sequences.

Similar doses of TNF (based on cytotoxicity on L929 cells) were added to myoblast cultures in the form of plasma membrane preparations of M9 cells or soluble recombinant TNF. The experiments proved that both soluble and transmembrane forms of TNF were able to induce the mobilization and assembly of C2/7 myoblast cells, producing identical aggregates (not shown).

TNF enhances the proliferating activity of the myoblast cells

The growth of myoblast cells in the presence of TNF was assayed by [^{3}H]thymidine incorporation and counting the cells. Exposure of myoblasts to TNF resulted in an increased proliferation of the cells (Tab. II). The myoblasts inside the aggregates remained alive and metabolically active for at least 10 days. However, their proliferation slowed down significantly, probably restricted by the limited availability of nutrients inside the aggregates.

TNF inhibits the expression of the genes of myofilament components: actin and myosin

The changes caused by TNF, higher cell densities (as a result of aggregation) and decreased proliferation of the aggregated cells could help myoblast differentiation, as both contact and cell cycle arrest are prerequisites of myotube formation.

As myogenic differentiation is characterized both in vivo and in vitro by the synthesis of muscle-specific structural proteins [3], we investigated the activity of corresponding genes in TNF-treated and control cultures. RNA samples from 1 to 8 day old cultures of C2/7 myoblast cells were purified and used to detect the expression of specific myogenic genes, skeletal actin, myosin heavy chain and myosin light chain by Northern analysis.

As shown in Figure 2, TNF exerted an unexpected inhibition on the synthesis of muscle-specific transcripts. While in control cultures the expression of actin and myosin genes was upregulated from day 3, simultaneously with the start of the fusion process, TNF treatment caused a marked inhibition of muscle-specific mRNA synthesis in the cells.

Electron microscopic studies showed that organized myofilaments were only visible in control cells. TNF-treated cells, probably as a result of the low-level expression of the myofibrillar proteins, contained no organized filaments (Fig. 3).

Tab. II. The effect of TNF treatment on the proliferation of myoblasts.

Days	TNF 15 ng/ml	50 ng/ml	150 ng/ml
1	158 ± 31	180 ± 18	140 ± 22
2	128 ± 17	117 ± 26	115 ± 22
3	83 ± 14	78 ± 22	67 ± 19
10	56 ± 12	65 ± 20	34 ± 8

Subconfluent monolayers of C2/7 myoblast cells were exposed to the indicated concentrations of TNF and labeled with radiolabeled thymidine, as described in Materials and methods. Proliferation of TNF-treated cultures was calculated as percent of the control cultures at the corresponding days.

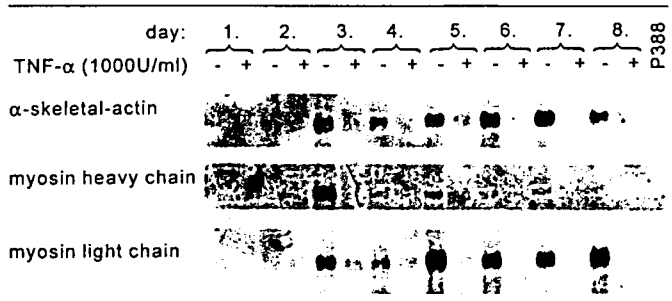

Fig. 2. TNF inhibited the expression of muscle specific genes. Expression of the genes of myofilament components in myoblast cells decreased in TNF-treated cells compared to that of controls. Myoblast cells were cultured on 24-well plates (10^5 cells/well). Fresh medium with or without 50 ng/ml TNF was added to culture medium on day 0, 4 and 7. Cell aggregates developed only in the TNF-treated cell cultures by the fifth day of treatment. Total RNA was extracted from TNF-treated and control C2/7 cells on every day of the experiment. Samples (20 μg) were separated by electrophoresis on a formaldehyde-agarose gel, blotted onto Hybond-N filters and hybridized with probes of alpha-skeletal actin, or heavy or light chains of myosin as described in Materials and methods. RNA extracted from P388 mouse myeloid leukemia cells served as negative control.

TNF inhibits the expression of the genes of myogenic regulatory factors

The synthesis of muscle-specific transcripts is controlled by myogenic regulatory proteins, which are activated during the differentiation process. Therefore, we investigated whether the inhibitory effect of TNF on actin and myosin genes is a consequence of a TNF-induced block or downregulation in the transcriptional activity of the genes of myogenic regulatory factors.

Northern analysis was used to detect changes in the expression of MyoD and myogenin genes. As myogenin and myf4 show high degree of homology, we used a probe of the human gene to detect the corresponding mRNA in mouse cells. As shown in Figure 4, TNF treatment inhibited the accumulation of transcripts of myogenic regulatory factors in C2/7 mouse myoblasts.

Myoblast cell aggregates contain collagen fibers

We observed a significant difference between collagen fiber content of TNF-treated and control cells. Collagen fibers can be seen in a number of electron microscopic pictures of TNF-induced myoblast cell aggregates (Fig. 5). Though the rate of collagen synthesis is increased during the cell-to-cell contact step of myogenesis [23] and according to literary data prefusion myoblasts synthesize type I, III and V collagen [1, 28], we could not detect any visible sign of collagen accumulation in the control cells undergoing normal myogenic differentiation.

TNF does not trigger the synthesis and release of TGF-β

In different target cells TNF is known to upregulate the synthesis of other cytokines and growth factors. To rule out the possibility that some of the changes were induced by TGF-β, we measured the level of the mRNA level of this growth factor in TNF-exposed and control myoblast cultures. According to our experiments TNF did not cause detectable changes in the transcription activity of TGF-β gene(s): both treated and control cells had very low levels of TGF-β messages (not shown).

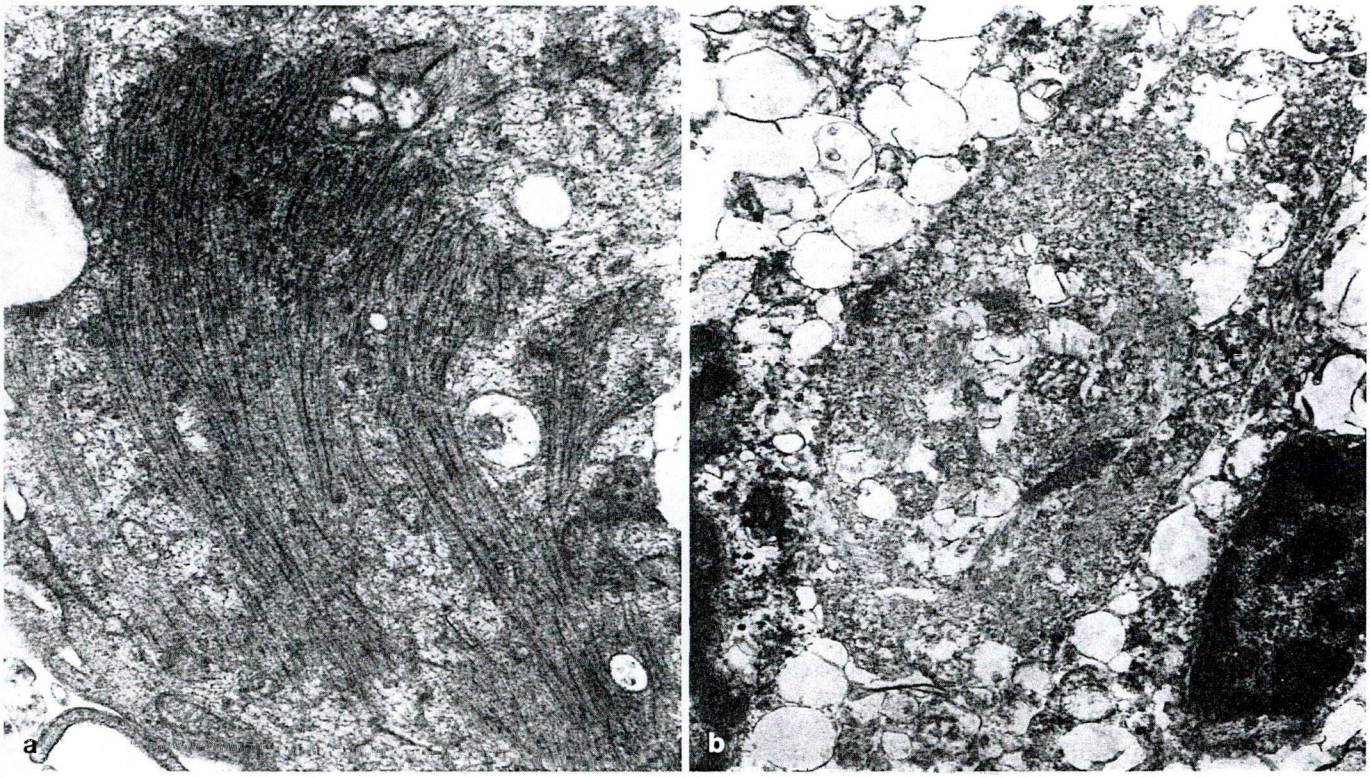

Fig. 3 Organized myofilaments are formed only in control myoblast cells. Electron microscopic picture of a 10 days old C2/7 myoblast culture (**a**) shows the presence of organized myofilaments, the sign of normal myogenic differentiation. In the cells of the aggregates of TNF-treated myoblast cells (**b**) similar structures cannot be observed. The culture shown in (**b**) was exposed to TNF for 10 days. – 40 000×.

Discussion

In order to learn about the role of TNF in developmental processes we investigated the effect of TNF in the differentiation process of C2/7 mouse myoblasts. This cell line was established from satellite cells of mouse thigh muscle after injury. Satellite cells are activated to form additional muscle cells during embryonic development, normal growth, wound healing and muscle regeneration [18].

The myogenic developmental process has four steps which can be followed easily in cultured C2/7 cells. It starts with the proliferation of myoblast cells until the adequate muscle mass is obtained. Myoblasts align and adhere to each other, and fusion starts. Fusion leads to the formation of myotubes, simultaneously extensive synthesis of the proteins of contractile myofilaments and other muscle cell specific proteins can be observed as a result of the activity of myogenic regulatory factors.

TNF treatment facilitated the first two steps of this process, myoblast proliferation was faster in TNF-exposed cells. TNF triggered a spectacular migration and aggregation of the treated cells within a few days. Further differentiation steps, however, were blocked by TNF treatment. The cytokine treatment inhibited the fusion of myoblast cells into multinucleated myotubules and blocked the accumulation of muscle cell-specific proteins. TNF-mediated inhibition of actin and myosin expression was reported earlier by Miller et al. [21] in human primary myoblasts. In our experiments synthesis of myogenic regulatory factors, MyoD and myogenin was also strongly inhibited by TNF treatment.

MyoD expression and activity are both inhibited by active cyclin D1-Cdk 4 complexes [14]. Cell cycle arrest, induced by p21 (and $p27^{KIP1}$) proteins ensures MyoD activity [14], which is normally strengthened by positive autoregulation [30]. It is possible, that the observed proliferative effect of TNF on myoblasts interferes with MyoD synthesis, and the subsequent block of differentiation is mediated by the increased activity of

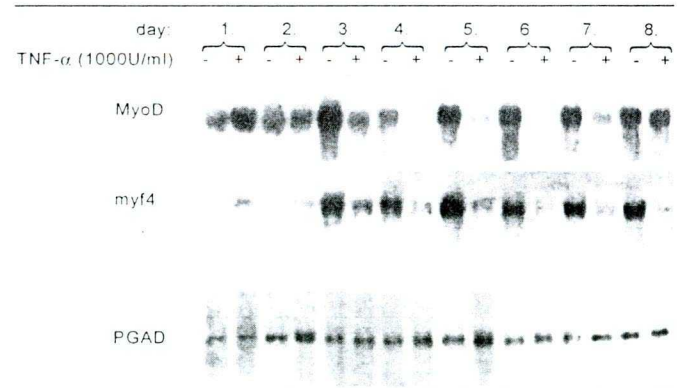

Fig. 4 The effect of TNF on the synthesis of transcripts of myogenic regulatory factors. Northern analysis of total RNA extracted from TNF-treated (50 ng/ml) and non-treated cells. Expression of myogenic regulatory factors MyoD and myogenin was strongly inhibited in TNF-treated cells. Transcripts of the same transcription factors were abundant in non-treated C2/7 cells.

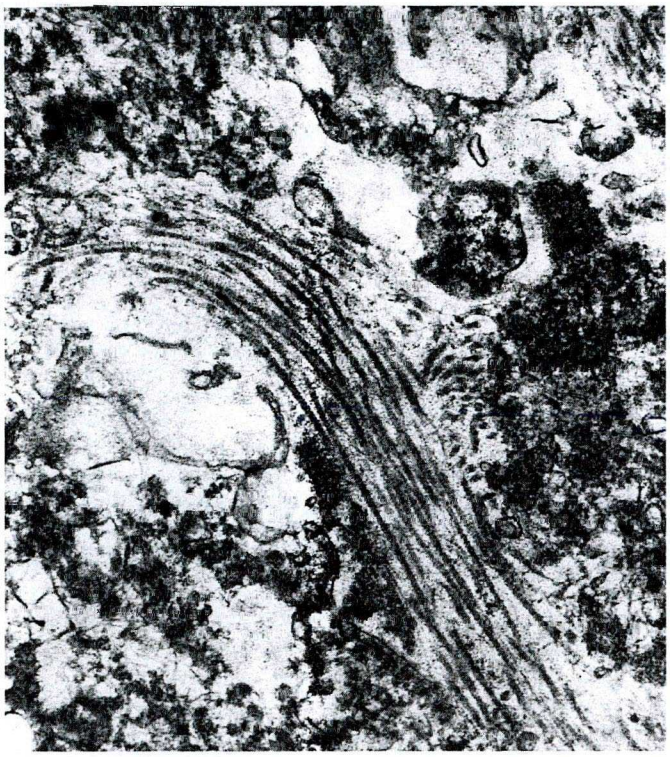

Fig. 5 Collagen fibers in TNF-treated myoblast cells. Collagen synthesis in C2/7 myoblast cells normally does not lead to the appearance of collagen fibers. This electron microscopic picture from a cell aggregate at the tenth day of culture shows collagen fibers characteristic for TNF-treated cells. – 40 000×.

cyclin-kinase complexes. Other transcription factors might also have a role: TNF-induced formation of (abnormal) myogenin-Jun-D complexes was reported in cachexia [5].

However, the presence of collagen fibers in TNF-treated myoblast cells suggested another explanation: TNF-induced TGF-β production. Collagen synthesis is almost always inhibited by TNF both in vitro [29] and in vivo [6]. TNF exerts a negative regulatory effect on collagen gene expression, antagonizing with TGF-β driven upregulation [9]. TGF-β, however, is documented to upregulate type I collagen expression and deposition in L6E9 rat myoblasts [16].

According to our measurements TGF-β message levels were very low, both in TNF-treated and control cell cultures, so it is unlikely that TGF-β is involved in the process.

Our results show, that TNF and its membrane-bound form are able to influence the differentiation of myoblast cells. Local (over)production of TNF as a result of stress or inflammation might lead to inhibition of muscle development, while systemic presence of TNF causes cachexia and muscle wasting [5]. This effect of TNF needs further studies as TNF produced during viral infection might contribute to embryonic malformations, while TNF-induced by ischemia and reperfusion could participate in myocardial cell loss in the afflicted areas.

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II.

45T-1, an established cell line with characteristics of Sertoli cells, forms organized aggregates in vitro after exposure to tumor necrosis factor alpha

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Tumor necrosis factor α (TNF) – Sertoli cell – differentiation – cell adhesion molecules

In the testis TNF is produced by germinal cells. The putative role of tumor necrosis factor alpha (TNF) in development and differentiation was investigated in 45T-1 mouse cell cultures, a cell line with characteristic markers of Sertoli cells, established from transgenic mouse families expressing the polyoma large T antigen in their testes. Exposure to TNF elicited a gradual assembly of the cells of the monolayer into highly organized spheroids. The first morphological sign of the changes was detected one week after TNF treatment by anti-desmin immunostaining which showed the formation of foci in the culture consisting of several hundred cells connected by an increasing number of cell contacts. Between days 10–20 the cells formed large ovoid or vermiform aggregates covered by several layers of flat, elongated cells. These cells extended septae into the inner mass of the spheroids consisting of loosely arranged, large polygonal or palisadic cells. The spheroids were surrounded by radially arranged elongated cells covered by small blebs. TNF treatment upregulated laminin expression in 45T-1 cell cultures, which is known to induce formation of cord-like structures by Sertoli cells in vitro. Coculturing 45T-1 cells with immortalized germinal cells or TNF-producing HeLa cells also lead to the formation of spheroids. These observations suggest that TNF production of germinal cells might contribute to the organization/differentiation of Sertoli cells.

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Abbreviations. BSA Bovine serum albumin. – DMEM Dulbecco's modified Eagle medium. – FCS Fetal calf serum. – FGF Fibroblast growth factor. – FGFR-1 Fibroblast growth factor receptor type 1. – FITC Fluorescein isothiocyanate. – FSH Follicle stimulating hormone. – IL-1β Interleukin 1β. – PBS Phosphate-buffered salt solution. – RER Rough endoplasmic reticulum. – SCF Stem cell factor. – SDS Sodium dodecyl sulfate. – TNF Tumor necrosis factor α. – WT1 Wilms' tumor gene product.

Introduction

TNF is a pleiotropic cytokine, which orchestrates the defensive forces of the immune system against invading microorganisms, viruses and malignant cells [1]. During the last few years TNF proved to be more than a cytotoxic cytokine. The presence of TNF and its transmembrane precursor form was detected during fetal and neonatal development of mice [15, 23]. Mouse blastocysts express the 55 kDa receptor of TNF [16] and both receptor types have been identified in mouse embryos [7, 10]. The importance of TNF activity during development was indicated by experiments where repeated injections of neutralizing antibodies to TNF into pregnant mice resulted in growth retardation of the fetus [11]. Studying the effects of TNF on different cell lines and their viral oncogene transformed derivatives we noticed morphological changes caused by TNF on different cell lines.

In preliminary experiments we have shown that TNF preserved the pluripotence of embryonic stem cells and inhibited the growth of trophoblast cells [21], induced a dedifferentiation-like process in embryonic chondrocytes [21] and inhibited the myogenic differentiation of myoblasts [22].

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These data suggest a possible role for TNF in normal development.

Here we report the effect of TNF on immortalized testicular cells, 45T-1, which exhibit markers of differentiated Sertoli cells. Sertoli cells play a crucial role in the proliferation, development and maturation of germinal cells [5]. Evidence is accumulating that paracrine and autocrine factors from Sertoli and germ cells are important in the functioning of both cell types [8]. Sertoli and germinal cells form the microenvironment of the seminiferous tubules, isolated from other cell types by a basement membrane and tight junctions of the Sertoli cells. As TNF receptor p55 was detected on Sertoli cells [13], spermatogenic cells express TNF [3] and we have shown the presence of the transmembrane form of TNF on mouse testicular germ cells [14], it was likely that TNF influences Sertoli cells.

Cell lines with Sertoli cell markers were established from transgenic mouse families, which carry an immortalizing viral oncogene, the polyoma virus large T gene under the transcriptional control of the viral early promoter [17]. Northern blot analysis detected the expression of the transgene only in the seminiferous epithelium of males. The transgenic animals develop normally and are fertile, but the elderly animals suffer from testicular tumors of Sertoli cell origin.

Cell line 45T-1 was established from the tumor of an old animal. These immortalized Sertoli cells have been growing in vitro for generations and their ability to nurse spermatogenic cells was further characterized [20]. They express a whole range of differentiated Sertoli cell markers, like steel factor (or stem cell factor, SCF, the ligand of *c-kit)*, WT1, transferrin and the alpha subunit of inhibin.

Materials and methods

Tissue culture

45T-1 cells, expressing Sertoli cell markers, derived from testicular tumor tissue of a mouse expressing the polyoma large T antigen in its gonads [17], were cultured in DMEM (SERVA, Heidelberg/Germany) containing 10% FCS (Inst. Jacques Boy, Reims/France) at 32°C. The cells were grown in vitro for many generations without noticeable changes in their growth characteristics. The cells exhibit anchorage dependence and contact inhibition. Confluent cultures can be maintained for several weeks without significant loss of viability, though sometimes the senescent cell layer peels off the substrate. No organization or aggregation of the cells can be observed without the use of substrates like Matrigel. In the reported experiments no such substrates were used.

GC-1 cells (ATCC CRL-2053) are mouse testicular cells immortalized by the large T antigen of SV-40. They show characteristics of a stage between type B spermatogonia and primary spermatocytes. HeLa cells (ATCC CCL 2) are human cervical carcinoma cells, M9 is a genetically modified HeLa clone transformed with a DNA construct coding for a slightly modified human TNF protein (with increased resistance to proteolytic processing). The transmembrane form of TNF accumulates on the surface of these cells [19]. GC-1, HeLa and M9 cells were grown in DMEM with 10% serum.

TNF and anti-TNF antibodies

Human recombinant TNF alpha was produced in *E. coli* cells (Tóth et al, Hungarian patents #: 5578/88 and 2030/90). The protein was purified to apparent homogeneity. Its purity was over 95 percent, showed one single band in silver-stained SDS gel electrophoretic analyses, had a specific activity of 20–50 U per ng protein and contained less than 0.05 ng endotoxin per mg protein.

The following anti-human TNF alpha antibodies were used: *a)* serum-free culture supernatants of B.154.7.1 hybridoma cells (generous gift of Prof. É. Gönczöl, Institute of Microbiology, A. Szent-Györgyi Medical University, Szeged/Hungary) producing a neutralizing monoclonal antibody were purified and concentrated by ammonium sulfate precipitation. 1 µl of the dialyzed, sterile filtered preparation neutralized 1 µg (> 2×10^4 U) TNF in 1 ml medium if incubated for 30 min at 37°C before titration. *b)* hyperimmune rabbit serum was purified on Pharmacia protein G (Pharmacia, Uppsala/Sweden) and immobilized on TNF columns. 10 µl of the dialyzed, sterile filtered preparation protected L929 or WEHI cells against the cytotoxic effects of 1 µg (> 2×10^4 U) TNF (incubated in 1 ml medium for 30 min at 37°C before titration) in a standard microplate assay (3×10^4 cells, 200 µl medium per well).

Microscopy and immunohistochemistry

45T-1 cells were grown in 24-well tissue culture plates. Cell monolayers and aggregates were fixed in 4% formaldehyde solution for 1 hour at 4°C. The fixed cultures were embedded in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN/USA) and frozen in petroleum ether cooled on a liquid nitrogen bath. Sections were cut at 15–20 µm in a cryostat, mounted on 0.4% gelatine-coated glass slides and dried overnight at 4°C. Sections were stained using chresyl violet.

For immunostaining cells were grown on glass coverslips, fixed and permeabilized in methanol at –20°C for at least 2 hours. After PBS (pH 7.4) washing the unspecific binding sites were covered in 2% BSA (bovine serum albumin, fraction VI., Sigma, St. Louis, MO/USA) for 60 min. Antibodies against mouse desmosomal protein, vinculin, cytokeratin CK5, vimentin and laminin (all from Sigma, St. Louis, MO/USA) were applied on the coverslips in 50-fold dilution (prepared in PBS, containing 0.2% BSA). Anti-alpha and beta tubulin and anti-cytoskeletal actin (both from Amersham, Buckinghamshire/England) were used in 1:100 dilution. Incubation times were 60 min at room temperature or overnight at 4°C.

In each immunohistochemical experiment the following controls were applied: samples incubated *1.* without first antibody (to check the unspecific binding of the second antibody), *2.* in the presence of non-immune serum (to prove the specificity of the immune reaction) and *3.* without the second antibody (to exclude the possibility of autofluorescence). After washing with PBS the cells were incubated in the presence of FITC or rhodamine-labeled, 100-fold diluted second antibody (Sigma, St. Louis, MO/USA) for 60 min at room temperature. On some samples the nuclei were counterstained in 0.001% ethidium bromide, then the coverslips were covered in PBS-buffered glycerol, containing a few crystals of para-phenylene-diamine and examined under an Axioscope microscope (Carl Zeiss, Germany).

Fluorescent labeling of cells and laser scanning microscopy

45T-1 cells were labelled with 25 µM of the fluorescent rhodamine derivative Cell Tracker Orange CMTMR, (Molecular Probes Europe BV, Leiden/ Netherlands) according to the manufacturer's recommendations. The labelled 45-T1 cells were cocultured with GC-1 cells (in different ratios) on Labtek glass slide chambers (Miles Scientific, Naperville, IL/USA). We kept the cells under growth condition for two weeks in the present of 10^3 U/ml TNF. The spheroids were observed in PBS under a confocal laser scanning microscope (Carl Zeiss, Oberkochen/Germany).

Transmission and scanning electron microscopy

For electron microscopic investigations samples from each experimental group were fixed in 2.5% glutaraldehyde containing 1% sucrose and 2 mM $CaCl_2$, buffered by 100 mM cacodylate, pH 7.0, for 1 h at room temperature. After washing in cacodylate buffer they were post-fixed in 1% osmium tetroxide and stained in 1% uranyl acetate. The samples were dehydrated in ethanol and propylen-oxide and embedded in Araldite (Durcupan, Fluka, Buchs/Switzerland). Ultrathin sections were stained by lead citrate and examined in a JEOL CXII electron microscope (JEOL, Japan).

For scanning electron microscopy the cultures were fixed in 1.5% glutaraldehyde, 0.5% formaldehyde, 1% sucrose, 2 mM $CaCl_2$ in 100 mM cacodylate buffer, pH 7.0, for 1 h at room temperature. The

specimens were postfixed with osmium tetroxide and dehydrated through a graded series of alcohol and critical point dried in a Sorvall type critical point dryer. After drying, the specimens were mounted on copper stubs and coated with gold-palladium and examined in a JEOL JSM 50 (JEOL, Japan) scanning electron microscope.

Results

45T-1 cells are resistant to the cytotoxic effect of TNF but exposure of monolayers to TNF leads to the formation of cell aggregates

TNF induces self destructive processes in certain tumor cells. However, 45T-1 polyoma large T-immortalized Sertoli cells showed a remarkable resistance against the cytotoxic effect of TNF, tolerating up to 2–3 μg/ml (10^5 U/ml) TNF without any loss of viability.

When near confluent cultures of 45T-1 cells were exposed to 0.2–200 ng/ml (10 to 10^4 U/ml) TNF at 32 °C for 6–24 h the cells did not show any cytopathic effect or morphological changes. However, after prolonged periods of time (3–15 days) the presence of TNF induced marked changes in their shape and morphology. First small, elevated foci of aggregated cells began to appear between the well spread and extremely flat cells characteristic for control cultures. They were surrounded by radially arranged elongated cells covered by numerous small blebs.

When cells were exposed to TNF for prolonged periods of time (7–15 days) they formed plaques, ridges and doughnut-shaped formations of multilayer structure. More and more cells joined these dynamic formations and, since the proliferation of the cells was very limited, large surfaces of the culture plate were emptied by the cells. After 12–20 days practically all cells aggregated into one or several spheroids, a few mm in size.

In previous experiments (Mohari and Duda, unpublished) we found that germinal cells produce several hundred units of the transmembrane form of TNF per million cells. Based on this figure, we calculated that subconfluent Sertoli cell monolayers (2×10^4 cells per cm^2 in 0.5 ml) should be exposed to approximately 2–20 ng/ml (40–400 U/ml) TNF to imitate the natural environment.

Experimentally 5 ng/ml (100 U/ml) was the lowest concentration, which induced morphological changes. Exposure of the cells to 1000 U/ml speeded up the process, but further increases in TNF concentrations did not shorten the time necessary for the formation of the aggregates. In all the following experiments 1000 U/ml TNF was used to induce the formation of spheroids.

There were characteristic changes in the shape of TNF-treated cells revealed by scanning electron microscopy. The flat, smooth surface, characteristic of 45T-1 cells grown in monolayers changed several days after TNF treatment. The cells developed blebs and protrusions as they assembled and formed aggregates (Fig. 1).

Most of the cells inside the TNF-induced aggregates remained alive and metabolically active even in six-week-old cultures. Protein synthesis, detected as labeled methionine incorporation, and mitochondrial activity, assayed as reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO/ USA), was maintained, though at a reduced level. (As restricted access of inner cells to nutrients made these measurements quantitatively unreliable, data are not shown.)

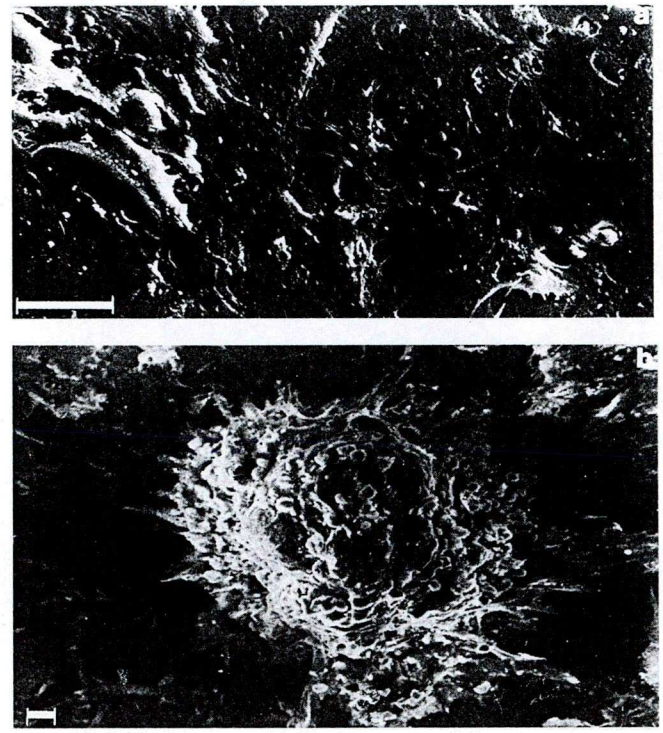

Fig. 1. Scanning electron microscopy of 45T-1 Sertoli cells. **a**: The monolayer of 6-days-old control cultures consisted of flat, polygonal cells. **b**: A spheroid formed under the effect of TNF (1000 U/ml, 10 days). Bars: 100 μm.

Microscopic investigation of intact aggregates showed tubular inner structures. Frozen sections revealed ordered organizations of the cells into garland-like formations (Fig. 2a). No aggregation or differentiation was observed in control cultures of 45T-1 cells in the absence of TNF. The cells grew into multilayered cultures if kept for long periods of time and showed no differentiation or inner organization in cross sections (Fig. 2c).

In well developed spheroids three structural domains could be distinguished by light (Fig. 2a) and electron microscopy (Fig. 2b): *1)* an outer layer, consisting of sheets of long, flat cells; *2)* bundles of onion skin-like sheets of elongated cells protruding from the periphery into the core and *3)* the inner mass of cells divided into more or less separated compartments by the bundles. The cells of the outer layer and the bundles were characterised by a prominent cortical network of microfilaments, ovoid nuclei, well developed RER and numerous cell contacts (Fig. 3a-c). The inner mass consisted of rather polymorphic cells among which large, round forms and tall, compactly arranged ones were equally found. Nuclei with deep invaginations were common in these cells and they contained lipid droplets as a rule. Apoptotic cells and intracellular spaces filled with cell debris were also observed in this area.

Immunohistochemical investigations of TNF-treated cells

Sertoli cells participate in the secretion and maintenance of basal membranes surrounding the seminiferous tubules. Production of laminin and collagen, components of the basal membrane, is a characteristic feature of differentiated Sertoli cells. On the other hand, extracellular matrix was shown to induce cord formation in primary cell cultures [9] and Matrigel was reported to have similar effects on a polyoma T antigen-

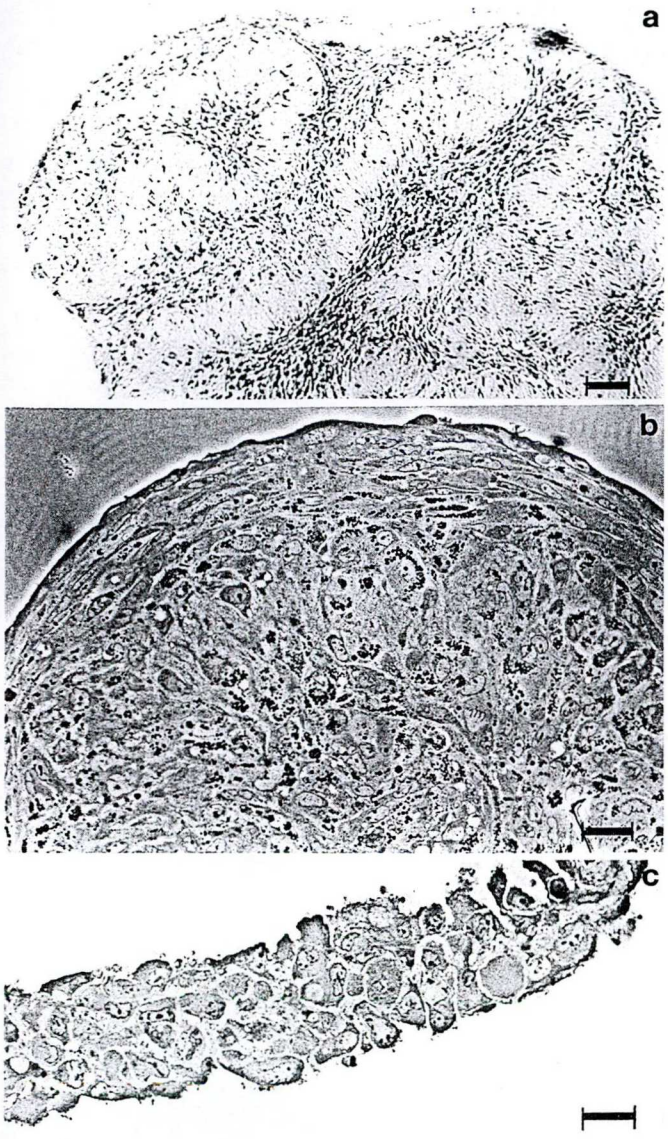

Fig. 2. Sections of frozen (**a**) and aldehyde-fixed, Araldite embedded (**b**) spheroids from cultures exposed to TNF (1000 U/ml) for 21 days. The spheroids are covered by several layers of flat, elongated cells which protrude into the core and divide the inner cell mass into more or less separated areas. The cells contain many lipid droplets. Control cells (**c**) were kept for 3 weeks in DMEM + 10% FCS, changing the medium twice weekly. Unlike TNF-treated cells, most control cells died by the end of the second week. Spots of surviving cells were sectioned at an angle of approx. 45 degree, therefore showing more than one layer of cells. No sign of morphological changes is visible. Bars: 100 μm.

immortalized cell line, very similar to 45T-1 [17]. We used anti-laminin antibodies for the detection of laminin expression in TNF-treated and control cultures. According to the immunohistochemical results expression of laminin was easily detectable in radially organized, aggregating cells of the monolayer 3 days after TNF exposure and highly upregulated in the spheroids at 10–12 days (Fig. 4a and b). Laminin-specific immunostaining was negative in control cultures and in non-aggregated cells of TNF-treated Sertoli cell monolayers (Fig. 4c).

The formation of the spheroids from monolayered cells requires changes in the general shape of the cells, reorganization of cell-cell and cell-matrix contacts. Aggregation involves migratory movements. All these events and changes are coordinated and deeply influenced by the cytoskeletal elements. Therefore, the presence and distribution of some cytoskeletal components (alpha- and beta-tubulin, cytoskeletal actin, cytokeratin, vinculin and vimentin) were investigated by immunohistochemical methods during the period of the TNF treatment. All of these proteins were present in both the control and TNF-treated cells. TNF treatment did not cause dramatic changes in the quantity and distribution of these proteins (not shown).

In control cultures and in the ones treated with TNF for 24 hours desmosomal protein was weakly expressed (Fig. 5a). However, 3 days after TNF exposure some of the cells became desmin positive and these cells were arranged in small patches in the cellular lawn (Fig. 5b). Six days after TNF treatment when the cells started to assemble into large aggregates and in the fully developed spheroids immunohistological examination proved the high level expression of desmin (Fig. 5c and d) and electron microscopic pictures also revealed the presence of numerous cell-cell contacts, especially between the cells of the outer layers of the spheroids (Fig. 3b).

The role of TNF in the formation of ordered inner structures in the spheroids

Neutralizing anti-TNF alpha monoclonal antibody and rabbit polyclonal anti-TNF were used to prove that the changes were triggered by TNF. 0.5 μl monoclonal or 5 μl polyclonal anti-TNF preparations were used per each ml of medium containing 1000 U/ml TNF. TNF-containing media were incubated with antibodies for 30 min at 37 °C before added to the cells. Highly purified anti-TNF preparations contained enough antibodies to protect sensitive L929 cells from the cytotoxic effect of 10^4 U/ml TNF, ten times more than used in the differentiation experiments. The presence of neutralizing antibodies in the culture medium prevented TNF-induced aggregation of the cells. Conditioned medium of TNF-treated (but thoroughly rinsed) Sertoli cells did not induce any changes in TNF untreated 45T-1 monolayers. Continuous presence of TNF was not necessary for the development of spheroids and inner structures. We could not detect any differences in desmin or laminin production of cells exposed to TNF (1000 U/ml) only once for 3 days, or continually for 14 days.

The presence of different compounds known to interfere with the TNF-evoked signaling also inhibited the development of the formation of organized structures within the aggregated cell mass. Staurosporin, a potent inhibitor of protein kinase C was effective in concentrations of 10^{-8} M. In the presence of the cyclo-oxygenase inhibitor indomethacin and thiol compounds, which block eicosanoid metabolism and NF-κB activation, respectively, TNF was unable to induce the migration and aggregation of the treated cells. Another inflammatory cytokine and functional analog of TNF, interleukin 1β (IL-1β; 1 to 10 thousand U/ml) induced the aggregation of 45T-1 cells, however, no inner structure was observed in these aggregates.

Coculture experiments

To investigate the effect of cell surface TNF on Sertoli cell clustering we cocultured 45T-1 cells with other cells producing different amounts of TNF. Human TNF genes are not expressed in HeLa human cervical carcinoma cells, but a HeLa clone, M9 produces TNF at high levels. The transforming human TNF-α gene present in M9 cells was modified to

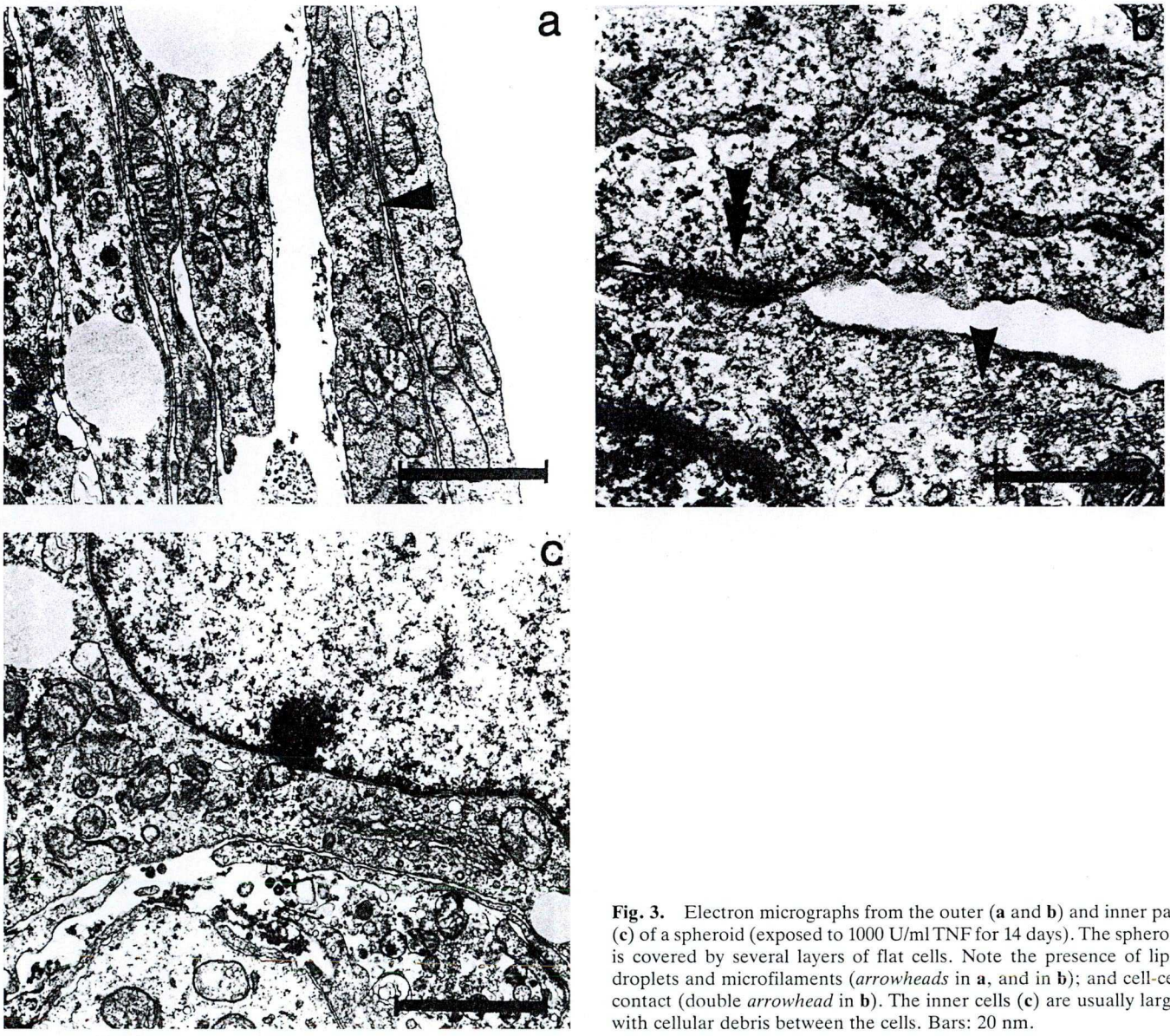

Fig. 3. Electron micrographs from the outer (**a** and **b**) and inner part (**c**) of a spheroid (exposed to 1000 U/ml TNF for 14 days). The spheroid is covered by several layers of flat cells. Note the presence of lipid droplets and microfilaments (*arrowheads* in **a**, and in **b**); and cell-cell contact (double *arrowhead* in **b**). The inner cells (**c**) are usually large, with cellular debris between the cells. Bars: 20 nm.

increase the resistance of preTNF to proteolytic processing [19]. As a result the 26 kDa, transmembrane form of TNF accumulates on the surface of M9 cells. HeLa cells did not influence the growth and morphology of 45T-1 monolayers at all. However, when 45T-1 cells were grown together with M9 cells for two weeks spheroids formed. The microscopic organization of these cell aggregates was very similar to the structures seen in TNF-induced spheroids (Fig. 6).

In other experiments GC-1, immortalized germinal cells, were cocultured with 45T-1 cells in vitro in the absence of external TNF. The presence of GC-1 cells could also elicit the formation of aggregates, suggesting that TNF produced by GC-1 cells was enough to induce clustering of Sertoli cells. Confocal microscopic studies of spheroids formed in cocultures of fluorescently labelled 45T-1 and unlabelled GC-1 cells showed that both cell types were present in the structures. (Proliferation of GC-1 cells (unlike 45T-1 cells) was not limited during the coculturing period and they usually overgrew 45T-1 cells and distorted cell patterns. Fluorescence of labelled cells decreased gradually and by the time spheroids formed it was impossible to record good quality images.)

These experiments prove that both soluble TNF and TNF present on the plasma membrane of neighbouring cells can elicit signaling events that lead to aggregation and differentiation-like changes in 45T-1 Sertoli cell cultures.

Discussion

We have shown that TNF, an inflammatory cytokine affecting the differentiation of several lineages of immune cells, was able to trigger characteristic morphological changes in cultures of a polyoma virus large T antigen-immortalized testicular cell line displaying marker proteins of differentiated, adult Sertoli cells. The cells of TNF-treated monolayers acquired migratory phenotype and underwent a gradual aggregation. This aggregation was accompanied by TNF-dependent formation of

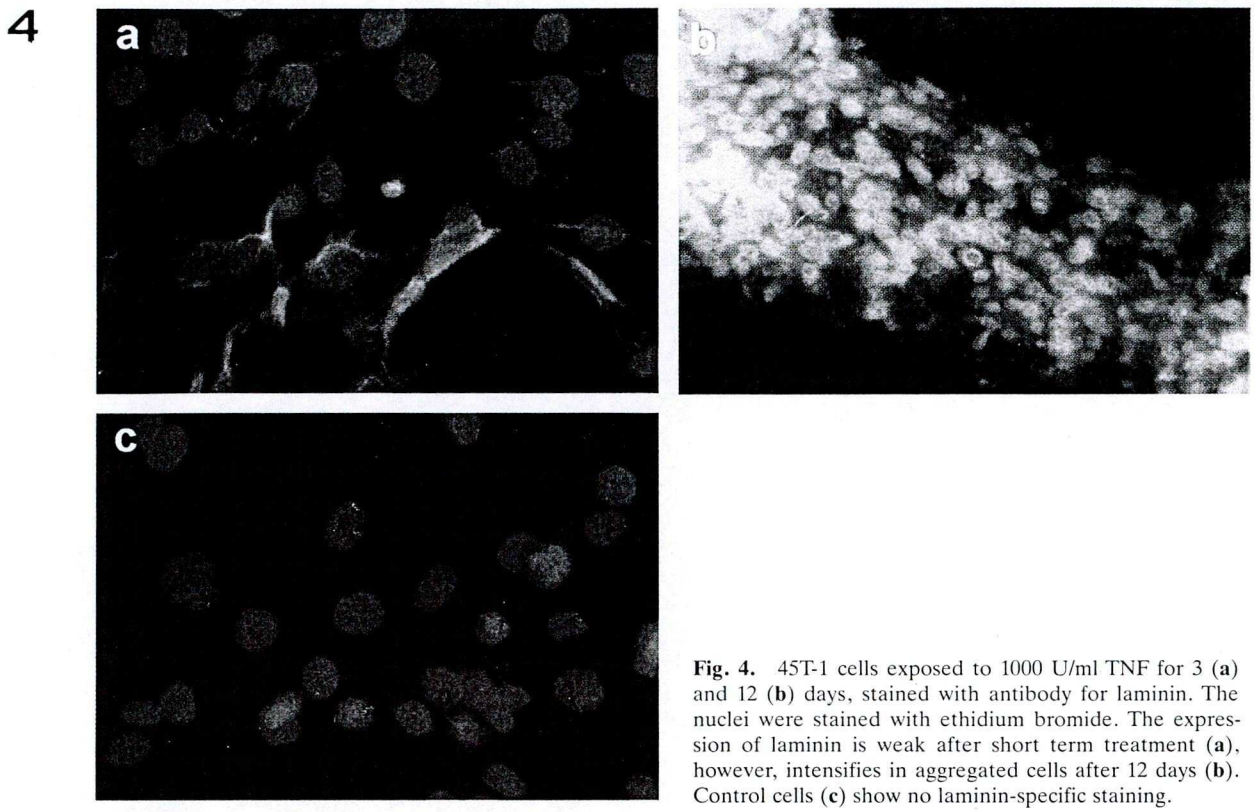

Fig. 4. 45T-1 cells exposed to 1000 U/ml TNF for 3 (**a**) and 12 (**b**) days, stained with antibody for laminin. The nuclei were stained with ethidium bromide. The expression of laminin is weak after short term treatment (**a**), however, intensifies in aggregated cells after 12 days (**b**). Control cells (**c**) show no laminin-specific staining.

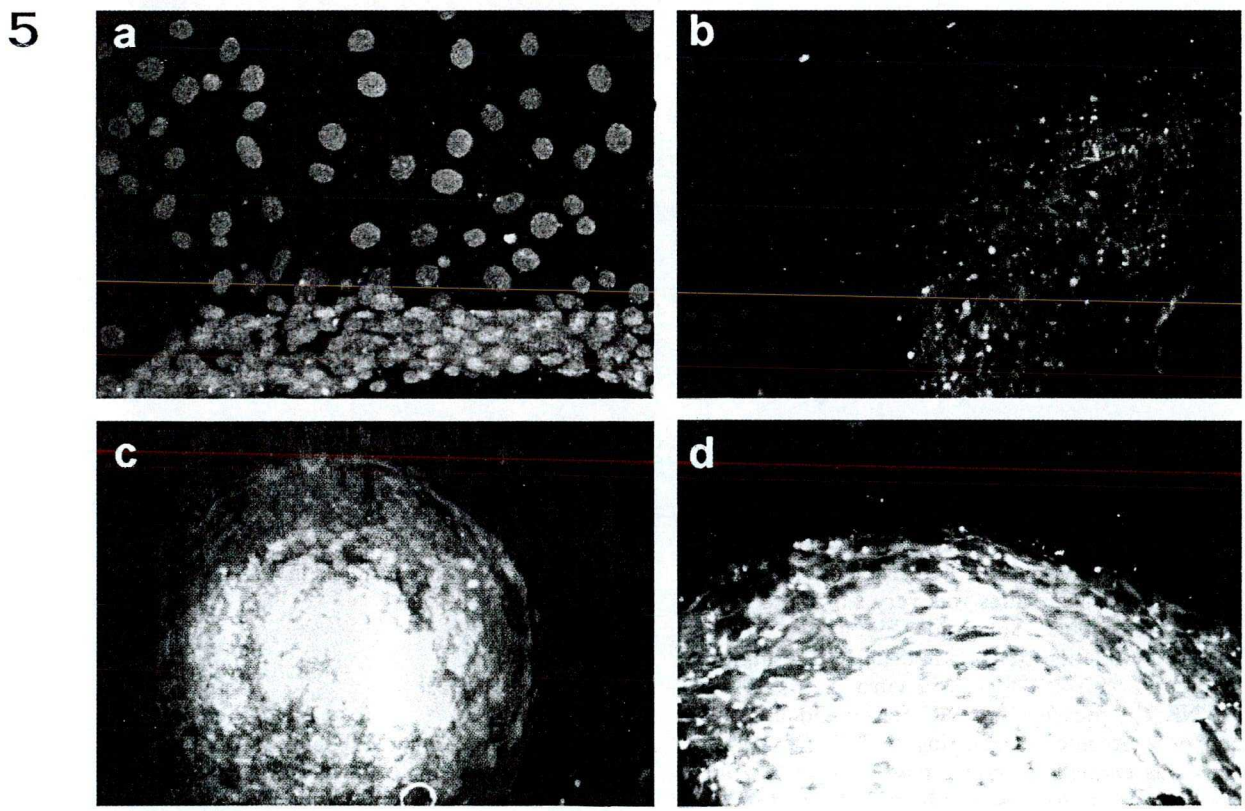

Fig. 5. 45T-1 cultures stained with antibody against desmosomal protein followed by FITC-conjugated secondary antibody as described in Materials and methods. The nuclei were stained by ethidium bromide. **a**: 3-days-old control cells; **b**, **c** and **d**: cultures exposed to 1000 U/ml TNF for 3, 6 and 12 days, respectively. Desmosomal protein-containing dots are prominent in the aggregating cells (**b**) and in well developed spheroids (**c** and **d**). The expression of the protein is weak in control cultures and in non-aggregated cells of TNF-treated cultures (around the spheroids, **b** and **c**).

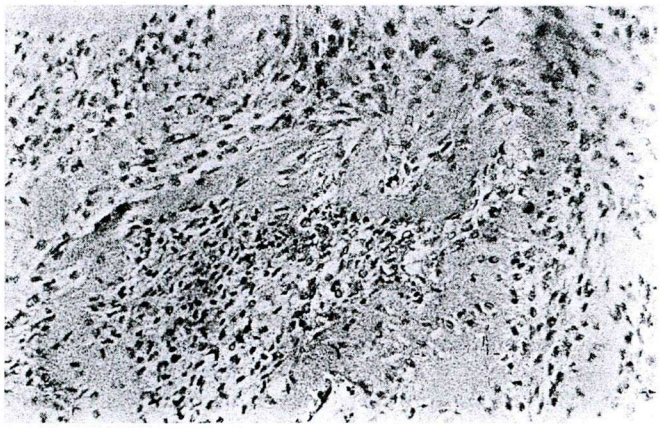

Fig. 6. Spheroids formed in cocultures of 45T-1 cells and M9 cells producing the transmembrane form of TNF (14 days of culture). TNF produced by M9 cells induced the formation of spheroids, mixed aggregates of 45T-1 and M9 cells. – × 100.

organized inner structures and upregulation of desmosomal protein and laminin detected by immunohistochemistry. While aggregation was also induced by the functionally related cytokine, IL-1β, organization of the inner structures was triggered only by TNF. Laminin production was highly upregulated in TNF-treated 45T-1 cultures. As laminin was reported to induce the formation of cord-like structures in Sertoli cell cultures in vitro [9, 17], it is quite possible, that accumulation of TNF-induced laminin provoked further steps of aggregation and differentiation of the cells.

Other findings [12] support the role of TNF in testicular development. It was reported that the type 1 receptor for basic fibroblast growth factor (bFGFR-1) was developmentally regulated in Sertoli cells, and bFGF action, when mediated by FGFR-1, is under the control of FSH, TNF and FGF. TNF was detected in spermatogenic cells [3] and the 26 kDa transmembrane form of TNF was present on the surface of germinal cells (Mohari and Duda, unpublished). This form of TNF shows characteristics of those membrane-bound, juxtacrine-acting growth factors that are supposed to play important roles in development and morphogenesis [2]. The presence of TNF alpha mRNA was detected by Northern blot analysis both in pachytene spermatocyte and round spermatid fractions. mRNA of the 55 kDa human TNF alpha receptor was present in Sertoli cells, but not in pachytene spermatocytes or round spermatids [3]. These results are consistent with the hypothesis that germinal cells produce TNF, which influences the metabolism of Sertoli cells and may play a role in the development and/or function of the germinal epithelium.

It is not totally unexpected that Sertoli cells form structures without the participation of germinal cells. In vivo tubule formation can also take place in the absence of germinal cells in *c-kit* receptor-deficient (white spotting) mice. Unlike homologous granulosa cells in the ovary, Sertoli cells retain many of their usual functions in these germ cell-free animals [4, 6]. This fact does not argue against the importance of TNF in development, as many other cell types (especially monocytes) are able to produce TNF. Normal development of TNF receptor null mutant mice [18] seem to argue against the role of TNF, but the activity of other members of the TNF receptor family of proteins could provide sufficient backup for the lost activity.

Coculturing experiments proved that TNF present on the surface of TNF-producing cells (including GC-1 germinal cells) can also induce the aggregation of 45T-1 Sertoli cells. Coculturing of different testicular cell types was described to lead to in vitro formation of different structures [5]. The fact that TNF can replace a cellular partner (or Matrigel) and induce 3-D organization of Sertoli cells suggests that the signaling pathways TNF triggers are similar to or identical with those elicited by germinal (or other testicular) cells and/or extracellular matrix components.

Summarizing, TNF induces a differentiation-like process in cultures of 45T-1 mouse Sertoli tumor cells, a phenomenon which could reflect the in vivo role of TNF in the development or regulation of seminiferous tubules.

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III.

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p53 is involved in the p120E4F-mediated growth arrest

Running title: The growth inhibiton by p120E4F is p53-dependent

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Abstract

Control of cell growth and division by the p53 tumor suppressor protein requires its abilities to transactivate and repress specific target genes and to associate in complex with other proteins. Here we demonstrate that p53 binds to the E1A-regulated transcription factor p120E4F, a transcriptional repressor of the adenovirus E4 promoter. The interaction involves carboxy-terminal half of p120E4F and sequences located at the end of the sequence-specific DNA-binding domain of p53. Ectopic expression of p120E4F leads to a block of cell proliferation in several human and murine cell lines and this effect requires the association with wild-type (wt) p53. Although p120E4F can also bind to mutant p53, the growth suppression induced by overexpression of the protein is severely reduced in a cell line that contains mutant p53. These data suggest that p120E4F may represent an important element within the complex network of p53 checkpoint functions.

Keywords: E4F/p53/growth suppression/transcriptional repression

Introduction

The product of the p53 tumor suppressor gene plays a pivotal role in controlling the onset and progression of human neoplasias, as is underlined by the high incidence of p53 mutations found in a broad spectrum of human cancers (Hollstein *et al.*, 1991). The central role of p53 as the safeguard of the genome integrity arises from its ability to respond to a variety of events that can lead to uncontrolled proliferation (for review see Giaccia and Kastan, 1998; Ko and Prives, 1996; Levine, 1997).

Several types of DNA damage, as well as other stress conditions, like hypoxia or ribonucleotide depletion, activate p53 leading to the transactivation of target genes which mediate, at least in part, p53-dependent growth arrest and apoptosis (An *et al.*, 1998; Giaccia and Kastan, 1998; Graeber *et al.*, 1996; Linke *et al.*, 1996). Activated p53 can also repress the transcription of various genes and this function may also contribute to its downstream effects (Murphy *et al.*, 1996; Wang *et al.*, 1997). These two functions of p53 may be mechanistically distinct. Transcriptional activation occurs through the direct binding of p53 to specific promoter elements, whereas repression may additionally require the association with other promoter-bound transactivators or repressors (Murphy *et al.*, 1996; Ori *et al.*, 1998).

The overall complexity of p53-dependent functions requires association of the protein with multiple cellular factors (Haupt *et al.*, 1995; Ruaro *et al.*, 1997; Wang *et al.*, 1996). Some of them act on p53 to modulate its functional activation in response to various signals (Giaccia and Kastan, 1998), to alter its DNA binding (Jayaraman *et al.*, 1997) and transcriptional activities (Gu *et al.*, 1997; Levine, 1997; Lill *et al.*, 1997) or its ability to modulate DNA repair (Wang *et al.*, 1995). In some other cases these proteins are targeted by p53 and the resulting complexes influence the efficiency of its checkpoint functions, as observed for the association of p53 with the XBP and XPD helicases, components of the TFIIH transcription/DNA repair complex, which is also involved in p53 mediated apoptosis (Wang *et al.*, 1996). The ability of p53 to associate with these cellular partners may be reduced or absent in transformed cells expressing mutant p53 (Iwabuchi *et al.*, 1994). In many cases, however, mutant p53 can still bind to these proteins, although the complexes are functionally inactive (Wang *et al.*, 1995).

In searching for new p53 interacting proteins, we isolated the transcription factor p120E4F (Fernandes and Rooney, 1997) in a yeast two-hybrid screen. p120E4F is one of two related zinc-finger proteins that differentially regulate the adenovirus E4 promoter in an E1A-dependent manner (Fernandes and Rooney, 1997). The full length protein, p120E4F, represses this promoter in the absence, but not in the presence of E1A. The other E4F protein, p50E4F is a proteolytically derived amino terminal fragment of p120E4F that was shown to stimulate the E4 promoter when coexpressed with E1A. Moreover, when ectopically expressed in mouse fibroblasts, p120E4F, but not p50E4F, blocked cell proliferation by inducing a cell cycle arrest near the G1/S transition (Fernandes *et al.*, 1998).

Here we demonstrate that E4F associates both *in vitro* and *in vivo* with p53 in mouse and in human cells. The association with p53 occurs through residues that are specific to p120E4F and is required for its growth suppression activity. p120E4F may therefore represent a novel p53 cellular partner that contributes to its checkpoint functions.

Results

Isolation of E4F as a candidate p53 associated protein

The yeast two-hybrid screen (Gyuris *et al.*, 1993) was employed to identify proteins able to associate with the p53H175 hot spot mutant. Approximately 3.5 million independent transformants were screened and twentyseven single clones were isolated and analysed for their ability to bind also wt p53 (LexAwtp53, aa 74-393). Sequence analysis revealed that one of the clones which interacted both with mutant and wt p53, corresponds to the carboxy-terminal half (starting from aa 350) of the E1A-regulated transcription factor p120E4F (Fernandes and Rooney, 1997). Structurally, p120E4F contains six zinc-finger motifs which are clustered in two separate regions: two motifs are contained within an amino terminal domain that is also present in p50E4F, while the remaining four are grouped within a central region (aa 436-568) found only in the full length protein (Fernandes and Rooney, 1997). The isolated clone (E4FΔ350) encodes the entire central zinc-finger domain and all further carboxy-terminal residues that are specific to p120E4F (Figure 1A).

To verify the interaction *in vitro*, the isolated cDNA was expressed in bacteria as GST-fusion protein (GST-E4FΔ350) and tested in a pull down experiment. Purified GST-E4FΔ350 or GST proteins were immobilised on GSH-Sepharose beads and incubated with *in vitro* translated p53H175, p53H273 and wt p53 proteins (Figure 1B). In agreement with the data obtained in the yeast two-hybrid system, p53wt, p53H175 as well as p53H273, were specifically retained on beads coupled with GST-E4FΔ350 (lane 7, 8 and 9). A specific interaction was also observed with *in vitro* translated mouse wt p53 protein (not shown). These data therefore demonstrate that E4F can directly associate with p53.

E4F associates with p53 in vivo

A longer clone, missing only the first 60 amino acids of p120E4F (E4FΔ60) was isolated from a human placental cDNA library and inserted into the expression vector pcDNA3, downstream of a START codon and a contiguous HA epitope (pcDNA3HA). This construct was used in transient transfection experiments to demonstrate the association between E4F and p53 within murine Balb/c Val 5 (Val 5) fibroblasts (Ginsberg *et al.*, 1991). This cell line stably expresses the temperature sensitive murine p53 valine 135 mutant, which exhibits a wild-type conformation at 32°C (permissive temperature) and a mutant conformation at 37°C (non-permissive temperature) allowing to directly test the binding affinity of E4F to both p53 forms within the same cells. Subconfluent growing cells maintained at 37°C were transfected with pcDNA3HAE4FΔ60 or with control plasmid (pcDNA3). Twelve hours after transfection, cells were split in two plates and incubated for 24 hours at the permissive or non-permissive temperature. Lysates of cells maintained at 32°C were immunoprecipitated with Pab 246, which recognizes wt p53, whereas extracts from cells grown at 37°C were immunoprecipitated with the mutant-specific antibody Pab 240. Immunoprecipitates were subjected to Western blot analysis with an antiserum raised against ΦAP3, the murine homologue of E4F (Fognani *et al.*, 1993). This antiserum has been shown to recognise both human and murine E4F proteins (Fernandes and Rooney, 1997). As shown in Figure 2A, ectopically expressed HA-E4FΔ60 associated equally well with wt and mutant murine p53 in Val5 cells (lane 4 and 8). Similar results were obtained when ΦAP3 was transiently overexpressed in the same cell line (Figure 2B, lane 4 and 8).

To confirm the association in human cells, the full length E4F cDNA (E4F2.5K; Fernandes and Rooney, 1997) cloned in pcDNA3HA, was transiently overexpressed in U2OS, a human osteosarcoma cell line expressing wt p53. Cell lysates were immunoprecipitated with the anti-p53 monoclonal antibody DO-1, then blotted with anti-HA antibody. As shown in Figure 2C, p53 associated with ectopically expressed p120E4F. Complex formation was also detected in cells transfected with pcDNA3HAE4FΔ350, a construct expressing E4FΔ350 (not shown).

Finally, to establish the existence of a complex of endogenous p53 and p120E4F proteins, cellular extracts from untransfected U2OS were immunoprecipitated with the E4F-specific antibody N4 (Fernandes and Rooney, 1997) or with preimmune sera as a control. Immunocomplexes were then resolved on SDS-PAGE and blotted for Western analysis with DO-1 antibody. As shown in Figure 2D, endogenous E4F was immunoprecipitated from U2OS cell lysates in complex with endogenous wt p53.

Taken together, these results clearly demonstrate that p120E4F can form a complex *in vivo* with wt and mutant p53, both in human and murine cells.

Binding to E4F requires amino acids 256 to 294 of p53

To identify the region of p53 involved in the interaction with p120E4F, we produced various wt p53 deletion mutants by *in vitro* translation and tested them for binding to GST-E4FΔ350 in a pull down experiment (Figure 3). As summarized in Figure 3B, E4F bound to all the C-terminally truncated p53 proteins used, however we observed significant differences in the efficiency of interaction. While the full length protein, capable of tetramerization, bound strongly to GST-E4FΔ350 (Figure 3A, lane 3), tr355, that forms dimers in solution (Tarunina and Jenkins, 1993) exhibited a weaker interaction (lane 6), suggesting that the quaternary structure of p53 may influence the association. Supporting this hypothesis, the two monomers, tr338 and tr298, are more impaired in their ability to bind to GST-E4FΔ350 (lane 9 and 12, respectively). The interaction with tr298 was very weak, suggesting that the E4F-binding region is located near to the end of the core domain (mapped between residues 102 and 292; Pavletich *et al.*, 1993; Bargonetti *et al.*, 1993; Wang *et al.*, 1993). In agreement with this assumption, a construct containing the tetramerization domain and the unspecific DNA-binding region but lacking the core domain (p53 Ct) was unable to interact (lane 18). An amino-terminal deletion mutant of p53 (p53Δ256), that overlaps 42 amino acids with tr298 and contains the tetramerization domain, interacted strongly (lane 15), demonstrating that binding to E4F requires amino acids 256 to 294 of human p53.

E4F does not affect the transactivation capability of wt p53

To test whether the transactivation activity of p53 was affected by the interaction with E4F, the p53-null SaOS-2 cells were transfected with the pG13CAT reporter plasmid (Kern *et al.*, 1992) together with vectors encoding for human wild-type p53 (p53 wt) and HA-tagged full length or amino terminally truncated E4F (HA-E4F2.5K and HA-E4FΔ350, respectively). An HA-tagged, carboxy terminal fragment of human p53 (p53 Ct; residues from 294 to 393) was used as a positive control, since a corresponding fragment derived from the murine protein has been shown to affect wt p53 functions in a dominant negative manner (Shaulian *et al.*, 1992). As shown in Figure 4A, neither HA-E4F2.5K nor HA-E4FΔ350 did significantly affect the transactivation ability of p53 (lane 4 and 5), whereas p53 Ct almost completely inhibited that function (lane 3). The expression levels of each proteins were comparable as judged by Western blotting (Figure 4B). The averaged results of three independent experiments are plotted in Figure 4C. We consistently observed a slight repression by overexpressing the full length E4F even when it was cotransfected with reporter constructs containing the SV40

or the thymidine kinase promoters in the absence of p53 (not shown). These findings therefore suggest that the interaction between E4F and p53 does not affect the transactivation capability of p53.

Binding to p53 requires amino acids 521 to 580 of E4F

The previous assays demonstrated that residues specific to p120E4F are required for p53 binding. To better define this region, several E4FΔ350 deletions were expressed as GST-fusions and used for pull down assay with *in vitro* translated wt p53 (Figure 5). Deletion of the last 89 amino acids (GST-E4FΔ350/NcoI) had no effect on p53 binding (Figure 5A, lane 4), while removal of residues 428-783, including the entire central zinc-finger domain (GST-E4FΔ350/SfiI), completely abolished the interaction (lane 6). A construct lacking amino acids from 552 to 783 (GST-E4FΔ350/PstI) was severly impaired in its ability to associate with *in vitro* translated p53 (lane 5), suggesting that residues in the last zinc-finger motif (comprising amino acids 548 to 568) might be involved in mediating the interaction. Indeed, two fusions that contain the intact last zinc-finger motif, GST-E4FΔ350-580 and GST-E4FΔ521 strongly bound to p53 (lane 7 and 9), while no interaction was detected with an E4F deletion lacking the entire zinc-finger domain and sequences upstream of it (GST-E4FΔ581, lane 8). Similar results were obtained when the different deletions were assayed in the yeast two-hybrid system (not shown). These data indicate that p120E4F contacts p53 through a carboxy-terminal region located between amino acids 521 and 580.

Ectopic expression of E4F requires wt p53 for growth suppression

Although cellular genes specifically regulated by E4F proteins are unknown, it was recently shown that p120E4F can mediate a growth arrest in NIH 3T3 cells, suggesting an important role for this protein in cell growth control (Fernandes *et al.*, 1998). In order to test the biological relevance of the complex formation between p53 and E4F, we overexpressed E4F in various human and mouse cell lines, harbouring wild-type, mutant or lacking endogenous p53.

Full length E4F (E4F2.5K) and various PCR-generated deletions were cloned in frame in pcDNA3HA vector (Figure 6A) and tested for their expression levels and subcellular localization. Transient transfections in U2OS cells revealed that all the constructs expressed similar amounts of proteins, as shown by Western blot analysis in Figure 6B. The subcellular localization of the translated proteins, assayed following microinjection by immunofluorescence with anti-HA antibody, is presented in Figure 6C. HA-E4F2.5K, as well as the carboxy terminal deletions HA-E4F/NcoI and HA-E4F/PstI, displayed heterogeneous subcellular distributions: some cells showed only nuclear localization whereas others revealed both nuclear and cytoplasmic staining. In contrast, HA-E4FΔ350, which lacks the amino terminal half of p120E4F, was exclusively nuclear.

To address the biological role of p120E4F, human cell lines expressing wt p53 (U2OS) or lacking p53 (SaOS-2) were transfected with pcDNA3HAE4F2.5K, pcDNA3HAE4FΔ350 or vector alone and tested for colony formation ability after two weeks of selection in G418-containing medium. The results of three independent experiments are shown in Figure 7A. A similar reduction (~50%) in colony numbers was observed both with HA-E4F2.5K and HA-E4FΔ350 in SaOS-2 cells as compared to the positive control empty vector (100%). In contrast, a much stronger growth suppression effect was detected when the E4F constructs were transfected into U2OS cells, thus suggesting the involvement of wt p53.

To more firmly establish the potential role of wt p53 in the p120E4F-mediated growth suppression, we microinjected a panel of murine cell lines expressing wt p53 (NIH 3T3) mutant p53 (Val 5, maintained at 37°C) or in which both p53 alleles are deleted (Balb/c (10)1) and tested the inhibition of S phase entry by bromodeoxyuridine (BrdU) incorporation assay. Asynchronously growing cells were microinjected as previously described (Del Sal *et al.*, 1992) with the various E4F constructs together with a plasmid expressing the green fluorescent protein (GFP) under the control of a CMV-promoter (pGFP-C1, Clontech) as a marker gene. Growth suppression in microinjected cells (GFP-positive) was measured by scoring the relative fraction of cells in S phase (anti-BrdU-positive) with respect to uninjected cells in adjacent fields (Del Sal *et al.*, 1992).

The results of at least four independent experiments are reported in Figure 7B. Enforced expression of HA-E4F2.5K blocked S phase entry in NIH 3T3 cells but neither in Balb/c (10)1, which lack endogenous p53, nor in Val 5 cells at the non-permissive temperaure. Similar results were observed with HA-E4F/NcoI and HA-E4FΔ350, both of them being able to associate with p53. Under the same conditions a similar growth suppression effect was observed by injecting wt p53 (not shown). In contrast, overexpression of the carboxy terminally truncated construct HA-E4F/PstI, which is deficient in binding to p53, did not inhibit S phase entry in any of the cell lines tested. In all the experiments performed, injection of the empty vector (pcDNA3HA) together with pGFP-C1 had no any relevant effect.

To further prove the involvement of wt p53 in the p120E4F-mediated growth suppression, Balb/c (10)1 cells were coinjected with HA-E4F2.5K or HA-E4F/PstI and limiting amount of p53 together with pGFP-C1 as marker and processed as described above. Overexpression of wt p53 alone resulted in a moderate growth inhibition (18%, Figure 7C) that was not affected by the coexpression of HA-E4F/PstI. Microinjection of HA-E4F2.5K alone had no any effect on cell growth, while coinjection of wt p53 and HA-E4F2.5K resulted in a 30% inhibition of S phase entry.

The results obtained by colony formation assays in human cell lines, together with the data from microinjection experiments in mouse cells, demonstrate that ectopic expression of p120E4F causes a growth suppression and cell cycle arrest that depends upon the presence of wt p53 and the ability of p120E4F to associate with it.

Discussion

In this report we demonstrated that the E1A-regulated transcription factor p120E4F associates with p53 and depends upon wild-type p53 to exert its growth inhibitory effect in human and murine cells.

Originally identified in HeLa cells as DNA-binding activities that recognise the adenovirus E4 promoter in an E1A-dependent manner (Raychaudhuri *et al.*, 1987), E4F factors are low abundance zinc-finger proteins that are expressed in a wide range of tissues (Rooney *et al.*, 1998). Although their cellular target genes are unknown, current evidence suggests that transcriptional regulation by E4F proteins may be somewhat complex. Within cells, two forms of E4F are present with distinct transcriptional activities. The full length p120E4F protein behaves as a transcriptional repressor of the E4 promoter in the absence of E1A, and introduction of the viral protein relieves the repression (Fernandes and Rooney, 1997) In contrast, p50E4F, a proteolytic fragment of p120E4F that contains the amino terminal zinc-finger domain required for E4 promoter recognition, is able to activate that promoter, but only in the presence of E1A (Fernandes and Rooney, 1997). E1A regulates the transcriptional activities of both forms of E4F by inducing their phosphorylation at critical residues involved in DNA-binding (Fernandes and Rooney, 1997; Fognani *et al.*, 1993; Raychaudhuri *et al.*, 1989).

Interestingly, ΦAP3, the murine homologue of E4F, is processed in a similar fashion (Fernandes and Rooney, 1997; Fognani *et al.*, 1993) and was originally isolated based upon its ability to bind to a completely different DNA sequence; a negative regulatory element within the adenovirus E1A promoter/enhancer (Fognani *et al.*, 1993). When overexpressed, ΦAP3 specifically repressed the transcription of a reporter gene controlled by that promoter (Fognani *et al.*, 1993). Moreover, whereas binding to the E4F consensus sequence requires only the amino terminal zinc-finger domain, recognition of the ΦAP3 site may occur through the central domain (Fernandes and Rooney, 1997; Fognani *et al.*, 1993; Rooney *et al.*, 1998). Thus, depending upon their proteolytic form and phosphorylation state, E4F proteins may participate in a number of independent transcriptional and physiological responses.

One physiological effect that occurs with ectopic expression of p120E4F in murine fibroblasts is a cell cycle arrest near the G1/S transition that correlates with a transcriptional-independent increase in the $p21^{Waf1,Cip1}$ and $p27^{Kip1}$ protein levels (Fernandes *et al.*, 1998). Notably, this effect was observed only with the full length p120E4F and not with the amino terminal form p50E4F, indicating a specific involvement of the carboxy-terminal half of the protein in this function.

In searching for p53-interacting molecules, we isolated several E4F clones in the yeast two-hybrid screening and all of them contained sequences encoding for the carboxy-terminal part of p120E4F, including the central zinc-finger domain. The binding site for p53 was localized to a region around the fourth zinc-finger motif. By coimmunoprecipitating wt and mutant p53 with transiently transfected E4F, or its murine homologue, ΦAP3, we demonstrated that the interaction occurs *in vivo* both in human and murine cell lines. More importantly, we have shown that endogenous p53 and p120E4F proteins also associate, suggesting that the resulting complex may play an actual role in cell proliferation or cell cycle control.

To establish the biological relevance of this interaction, we performed microinjection experiments and colony formation assays by using a panel of murine and human cell lines expressing wt or mutant p53 or lacking endogenous p53. In the microinjection experiments enforced expression of full length p120E4F or the short carboxy-terminal deletion E4F/NcoI inhibited S phase entry in cells harbouring wt p53, whereas expression of E4F/PstI, a deletion inefficient to bind to p53, had no effect. In contrast, no inhibition of S phase entry was observed in p53 null cells (Balb/c (10)1) or in cells expressing mutant p53 (Val5, 37°C). Overexpression of p120E4F greatly inhibited colony formation by human cells harbouring wt p53 (U2OS), although a 50% reduction was observed with p53 null cells (SaOS-2) as well. This type of assay, however, allows the detection of a growth suppression effect in its more general sense (growth arrest and/or apoptosis), therefore the 50% colony reduction achieved in SaOS-2 cells may reflect an involvement of p120E4F in a p53-independent mechanism that is inoperative in the transient microinjection assay. Nevertheless, the results clearly show that p53 and p120E4F interact and strongly repress cell proliferation.

One apparent discrepancy with previous results that should be noted, is that the role of p53 in mediating p120E4F growth suppression was initially excluded based upon the observation that cotransfection of a dominant negative p53 mutant with p120E4F had no effect on colony reduction in NIH 3T3 cells (Fernandes *et al.*, 1998). However, the design of those experiments did not take into account the ability of p120E4F to physically interact with wt and mutant p53. Thus, given the relative amounts of transfected p120E4F and mutant p53 constructs used by Fernandes *et al.*, it is likely that the excess levels of p120E4F were sufficiently high to titre out the mutant p53 protein and prevent its abrogation of wt p53 function.

p53 is a transcription factor that binds DNA in a sequence-specific manner with its central domain located between amino acids 102 and 292 (Pavletich *et al.*, 1993; Bargonetti *et al.*, 1993; Wang *et al.*, 1993). Here we demonstrated that p120E4F contacts p53 in its DNA-binding region *in vitro*, and residues from 256 to 294 of p53 are involved in mediating the interaction (Figure 3). Interestingly, we observed a correlation between the quaternary structure of p53 and its ability to associate with p120E4F, suggesting a complex regulation of protein-protein interaction. Although p120E4F binds to the end of the core domain of p53, this association does not affect the transactivation ability of p53 *in vivo* (Figure 4). This may be due to the fact that under the experimental conditions used, only a small fraction of p53 is bound to p120E4F, allowing unbound p53 to activate its reporter. Alternatively, the complex formation does not affect the DNA-binding capability of p53. Further investigations are required to distinguish between these possibilities.

Our evidence, that p120E4F can induce a p53-dependent block of cell proliferation without having an obvious effect on p53 transactivation function is consistent with the previous observation that elevated p21 protein levels in p120E4F-arrested cells occurred independently of an increase in p21 gene transcription (Fernandes *et al.*, 1998). This suggest that the p120E4F-mediated growth suppression may involve a number of target genes that are not directly regulated through consensus p53-binding sequences, rather through E4F or ΦAP3 sites.

We note that in both the colony formation and the microinjection assays, the truncated E4FΔ350 protein, which lacks the amino-terminal zinc-finger domain and therefore fails to bind the E4F consensus sequence (Fernandes and Rooney, 1997), was sufficient to induce growth inhibition both in human and murine cells. This suggests that cellular target genes of p120E4F responsible for the growth arrest effect may contain the ΦAP3 site. However, it is possible that p120E4F mediates this effect solely by bridging other proteins and may not require any DNA-binding function.

How p53 fits into this scenario is not yet clear. Control of gene expression generally requires the interaction of multiple factors, including several activators, coactivators, repressors and basal factors to stabilize the transcriptional machinery and determine its specificity. It is conceivable that p53 may act as a p120E4F-cofactor, to regulate the differential recognition of alternative DNA-binding sites for E4F proteins (Fernandes and Rooney, 1997; Fognani *et al.*, 1993) or the association of p120E4F with other factors in the transcriptional complex. Alternatively, E4F may be involved in modulating the transcriptional repression function of p53. Notably, although mutant p53 can still associate with E4F, the complex is not functional in terms of growth suppression. This indicates that the conformation of p53 in the complex is important for the E4F function, and may reflect a requirement to bridge p120E4F to other factors that recognize only wt p53. Several wt p53-binding proteins are also able to interact with mutant forms of p53, but, as with p120E4F, the mutant proteins lack the ability to regulate the activity of these partners (Wang *et al.*, 1995). The potential role of p53 as an E4F-cofactor may be particularly relevant when cells are exposed to stimuli which normally induce p53, since cells lacking the p53 checkpoint are seriously deficient in the p120E4F-dependent response.

Truncations at the carboxy-terminal end of p120E4F, which impair the binding of p53, nullify its growth inhibitory effect. Thus it would be of interest to look for the presence of genetic alterations in the E4F gene, mapped on chromosome 16p13.3 (Rooney *et al.*, 1998; Saccone *et al.*, 1998), within tumor cells that express wt p53.

A better understanding of the role of the p53/E4F complex requires characterization of other factors that associate with the carboxy half of p120E4F and most of all, the identification of E4F cellular target genes. Our evidence that p53 can physically associate with E4F and is required for its growth suppression effect, suggests that p120E4F is an important element within the complex network of p53 checkpoint functions.

Materials and methods

Cell lines and transfections

All the cell lines employed were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The Balb/c (10)1 cell line does not contain endogenous p53 (Harvey and Levine, 1991); the Val 5 cell line is a Balb/c (10)1 derivative, stably expressing the murine p53Val135 carrying a temperature-sensitive mutation at Valine 135, which behaves as mutant at 37°C and as wt at 32°C (Ginsberg *et al.*, 1991; Wu and Levine, 1994). Transfections were performed by the standard calcium phosphate precipitate method. For transient transfections cells were seeded 8-12 hours before transfection and further processed 24 to 36 hrs after removing the precipitate. CAT-reporter assays were performed by routine procedures. For colony formation assays cells were seeded at low density in 3 cm dishes and transfected with 1 μg of the indicated vectors. One day posttransfection cells were trypsinized and replated in 10 cm dishes in a medium containing 500 μg/ml geneticin (G418-sulphate, GIBCO). After two weeks of selection, the surviving colonies were fixed with 3% paraformaldehyde and stained with 10% Giemsa. The number of the empty vector-expressing colonies was scored as 100%.

Plasmids

To generate the LexA-fusion constructs, human p53H175 and wt p53 cDNAs were PCR-amplified between aa 74 and aa 393 and cloned in frame into pLexA202 (Gyuris *et al.*, 1993). To construct pcDNA3HAE4FΔ350, the E4F cDNA (from aa 350 to aa 783), isolated from the two-hybrid screening was inserted into pcDNA3 (Invitrogen) downstream to a START codon and a contiguous HA-epitope. To generate pcDNA3HAE4FΔ60, an E4F cDNA (aa 60 to aa 783) isolated from a human placental cDNA library in lambda ZapII (Stratagene) screened with E4FΔ350 was cloned into pcDNA3HA. pcDNA3HAE4F2.5K was generated by subcloning E4F2.5K from pCMVs-E4F2.5K (Fernandes and Rooney, 1997) into pcDNA3HA. pCMV-ΦAP3 has been already described (Fognani *et al.*, 1993). All the carboxy-terminal truncations of E4F as well as E4FΔ581 were obtained by PCR-amplification. For the GST-pull down assay E4FΔ581, E4FΔ350 and carboxy-terminally truncated derivatives were subcloned into pGEX4T-1 (Pharmacia) as EcoRI/XhoI fragments. E4FΔ521, a shorter carboxy-terminal fragment of E4F (aa 521-783), obtained from the placental cDNA library screening, was subcloned into pGEX4T-2 (Pharmacia) as a BamHI/XhoI fragment. To construct pcDNA3HAp53Ct, the human p53 fragment from aa 290 to 393 was PCR-amplified and cloned downstream of the HA epitope into pcDNA3. p53Δ256 was obtained from the yeast two-hybrid screen and subcloned into pcDNA3HA. The p53 truncations tr355 and tr338 (Tarunina and Jenkins, 1993) were cloned respectively in pBSK (Stratagene) and pSP6 (Promega). The truncation tr298 was produced by PCR and cloned into pBSK. pRcCMVhp53H175 and pRcCMVhp53H273 have been already described (Hinds *et al.*, 1990). pcDNA3wtp53 contains the full length, human wtp53 cDNA cloned as an EcoRI fragment in pcDNA3. The p53-reporter plasmid employed for CAT assays, PG13CAT, has been described previously (Kern *et al.*, 1992). All the PCR-amplified products were checked by sequence analysis.

Yeast two-hybrid screen

p53H175 fused to the LexA DNA-binding domain was introduced into the EGY48 (*MAT a trp1 ura3 his3 LEU2::pLEXAop6-LEU2*) yeast strain (Gyuris *et al.*, 1993), previously transformed with the pSH1834 plasmid, and selected on Ura- His- plates. This strain was transformed with a cDNA library from human WI38 fibroblasts made quiescent by serum starvation and contact inhibition cloned into the pJG4-5 plasmid (Gyuris *et al.*, 1993; Lamphere *et al.*, 1997). Approximately 3.5×10^6 primary yeast transformants were selected on plates Ura- His- Trp- and then pooled together. The interaction was performed by plating about 10×10^6 clones from the pooled library onto Ura- His- Leu- Trp- plates containing galactose. After three days, around 200 clones that grew and turned blue when subjected to β-gal expression assay were purified and further processed for the isolation of the plasmid. Plasmids containing the cDNA were selected in the E. coli B290 (Gyuris *et al.*, 1993) strain and classified through restriction analysis and Southern dot blot.

GST-pull down assay

The indicated Glutathione S-transferase (GST)-fusion proteins were produced in BL21 bacteria and purified by conventional procedures. Briefly, overnight cultures were diluted 1:10 and grown to OD_{600} =0.7, then the production of the fusion proteins was induced by the addition of 250 μM isopropylthiogalactopyranoside (IPTG) at 30°C for 4-6 hrs. The cells were lysed by sonication on ice in PBS containing 1% Triton X-100, 0.02 % SDS, 0.5% NP-40, 0.1% Tween-20, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml of each of chymostatin, leupeptin, antipain and pepstatin. The proteins were purified with Glutathione Sepharose 4B beads (Pharmacia) according to the manufacturer's instructions and the concentrations were estimated after SDS-PAGE. In the pull down assay 2 μg of GST-fusion protein or GST was incubated with ^{35}S-labelled *in vitro* translated protein (TNT-coupled reticulocyte lysate system, Promega) in 200 μl of binding buffer (150 mM NaCl, 20 mM HEPES pH 7.5, 10% glycerol, 0.05% NP-40) for 1 hr at 4°C. After washing, the bound complexes were solubilized with 2X Laemmli sample buffer and loaded onto an SDS-polyacrylamide gel. 25% of the amounts of *in vitro* translated protein used for the binding reaction was loaded as input. The bands were visualized by autoradiography.

Immunoprecipitation and Western blot analysis

Subconfluent cells seeded on 10 cm diameter Petri dishes were transfected with the indicated expression vectors by the conventional calcium phosphate procedure. 36 hrs posttransfection cells were washed with ice-cold phosphate buffered saline (PBS), then harvested in 1 ml of ice-cold lysis buffer as previously described (Muller *et al.*, 1997). The lysis was performed at 4°C for 30 minutes then the lysates were clearified by centrifugation. Two μg of purified antibodies or two ml of hybridoma supernatant (anti-p53 antibodies Pab 240 and Pab 246) prebound overnight to 20 μl of Protein A Sepharose CL-4B or GammaBind G Sepharose beads (both from Pharmacia) were then added to each sample and incubation at 4°C was continued for 6 hours. The beads were then washed three times in 1 ml of ice-cold lysis buffer and the bound proteins were solubilized by addition of 20 μl of 2X Laemmli sample buffer. Western blotting was performed according to standard procedures by using the following primary antibodies: 12CA5 (anti-HA, Boehringer Mannheim), anti-ΦAP3 (Fognani *et al.*, 1997),

DO-1 (anti-p53), N4 (anti-E4F; Fernandes and Rooney, 1997), anti-GST (Pharmacia). The bands were visualized by enhanced chemiluminescence (Amersham).

Microinjection, immunofluorescence and BrdU incorporation assay

Cells cultured in DMEM containing 10% FCS were grown on coverslips in 35 mm Petri dishes. After 24 hours of incubation at 37°C, cells were microinjected with the various plasmids by the Automated Injection System (Zeiss, Oberkochen, Germany) as described previously (Del Sal *et al.*, 1992). For expression analysis, microinjected cells were grown for 24 hours, fixed with paraformaldehyde (3% in PBS), then processed for immunofluorescence staining with 12CA5 anti-HA antibody (Boehringer Mannheim), followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG2b antibody (Southern Biotechnology Associates). For BrdU incorporation assay, cells were microinjected with the indicated expression vectors (50 ng/μl) together with 25 ng/μl pGFP-C1 (Clontech), a vector expressing the green fluorescent protein (GFP), used as marker. After microinjection, cells were grown in DMEM supplemented with 10% FCS for 20 hours then pulsed with 50 μM BrdU for four hours and fixed with paraformaldehyde (3% in PBS). After 20 minutes, coverslips were washed with PBS and incubated for 5 min in 0.1 M glycine-PBS. Permeabilization was performed with 0.1% Triton X-100 in PBS for 4 min. To reveal incorporated BrdU, coverslips were treated with 50 mM NaOH for 10 seconds and immediately washed three times with PBS. The GFP was used as coinjection marker since the NaOH-treatment affected the detection of the HA-tagged constructs by the 12CA5 anti-HA antibody. BrdU was revealed by anti-BrdU monoclonal antibody (Amersham) followed by incubation with rhodamine isothiocyanate (RITC)-conjugated anti-mouse IgG2a antibody (Southern Biotechnology Associates). Nuclei were counterstained with Hoechst 33342 (2 μg/ml in PBS) for 2 minutes followed by three washes in PBS. Coverslips were mounted with Mowiol mounting medium on a microscope slide and observed in a Zeiss confocal microscope with the appropriate filters.

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Figure legends

Figure 1. (**A**) Schematic representation of p120E4F and p50E4F proteins and the relative position of the E4F protein encoded by the cDNA isolated from the yeast two-hybrid screen (E4FΔ350). Numbers refer to amino acids. (**B**) E4FΔ350 binds to *in vitro* translated wt and mutant human p53. E4FΔ350 was expressed as GST-fusion protein (GST-E4FΔ350) and 2 μg of purified protein was incubated with ^{35}S-labelled wt (p53 wt) or mutant (p53H273 and p53H175) p53. A fraction of the input (25%) of *in vitro* translated products (lanes 1, 2, 3) as well as the GST (lanes 4, 5, 6) and GST-E4FΔ350 bound proteins (lanes 7, 8, 9) were resolved on SDS-PAGE and visualized by autoradiography.

Figure 2. E4F associates with p53 in mammalian cells. (**A**) Val5 murine fibroblast cells maintained at 37°C were transfected with 10 μg of pcDNA3HAE4FΔ60 (HA-E4FΔ60) or with empty vector (pcDNA3). Eight hours after removing the precipitates, cells were split in two plates and grown for 24 hours at the permissive temperature (32°C, p53 wt conformation) or at the non-permissive temperature (37°C, p53 mutant conformation). Immunoprecipitation was performed by using wt p53-specific antibody (Pab 246) on cell lysates obtained from cells maintained at 32°C and mutant p53-specific antibody (Pab 240) for cells grown at 37°C. Immunocomplexes were resolved on a 10% SDS-polyacrilamide gel and subjected to Western blot analysis with anti-ΦAP3 antiserum that recognizes both human and murine E4F proteins. (**B**) Val5 cells were transfected with 4 μg of pCMV-ΦAP3, encoding for the murine homologue of E4F (ΦAP3) or with the same amount of pcDNA3 then immunoprecipitation and Western blotting was performed as described for (**A**). (**C**) U2OS cells were transfected with 10 μg of pcDNA3HAE4F2.5K (HA-E4F2.5K) or pcDNA3 and p53 was immunoprecipitated with an amino terminus-specific antibody (DO-1). After SDS-PAGE, the blotted proteins were probed with anti-HA antibody. (**D**) Endogenous E4F was immunoprecipitated from U2OS cells with anti-

N4 antiserum (N4), specific for the amino terminus of E4F, or with preimmune sera (IgG) as control and immunocomplexes were analysed in Western blot by using the DO-1 antibody.
T, total cell lysate; **IP**, immunoprecipitation performed with the indicated antibody

Figure 3. Amino acids 256 to 294 of p53 are required for the association with E4F. (**A**) ^{35}S-labelled, *in vitro* translated full-length wt p53 and carboxy terminally truncated derivatives were incubated for 1 hour with 2 μg of GST-E4FΔ350 or GST prebound on GSH-Sepharose beads. The bound proteins were resolved on SDS-polyacrilamide gels (12.5%, left panel, 15% right panel) and visualized by autoradiography. (**B**) Schematic representation of the various p53 constructs used in the pull down assay and a summary of their binding properties to GST-E4FΔ350. All the numbers refer to amino acids. The functional domains of p53 are indicated.

Figure 4. E4F does not affect the transactivation function of wt p53. (**A**) 3_x10^5 SaOS-2 cells were cotransfected with 2 μg of pG13CAT, 100 ng of pcDNA3wtp53 (p53 wt) and 2 μg of either pcDNA3HAE4F2.5K (HA-E4F2.5K), pcDNA3HAE4FΔ350 (HA-E4FΔ350) or pcDNA3HAp53Ct (p53 Ct). 36 hours after transfection cell lysates were processed for CAT reporter assay. (**B**) Western blot analysis of an aliquot of the lysates used for the CAT reporter assay. HA-tagged proteins were revealed with anti-HA antibody (upper panel), while the amount of transfected p53 in each sample was controlled by using the DO-1 antibody (lower panel). (**C**) Graphic plot representing the mean from three independent experiments. Values are reported as percentage of conversion and were obtained with a Packard Instant Imager.

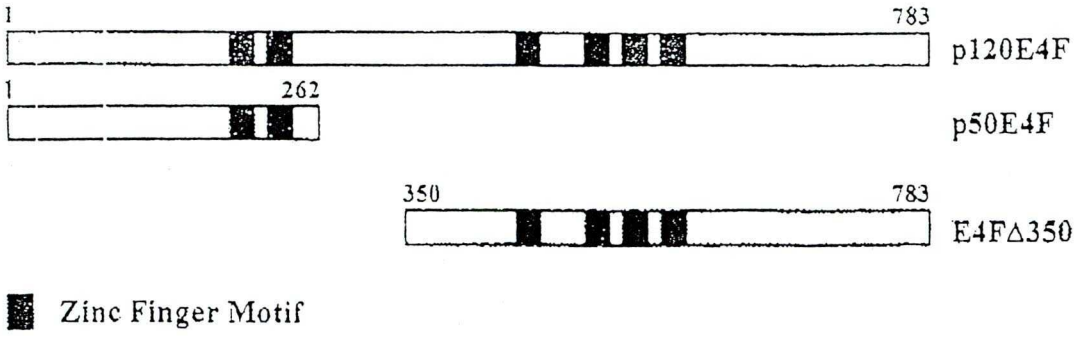
Figure 5. The interaction between E4F and p53 occurs at the carboxyl terminus of p120E4F. (**A**) GST-pull down assay was performed as described for Figure 3A with *in vitro* translated wt p53 and the various E4F deletion constructs expressed as GST-fusion proteins. (**B**) Expression pattern of the deletion constructs analysed by Western blot on same amount of purified proteins with anti-GST antibody. (**C**) Schematic representation of the various E4F truncations expressed as GST-fusion proteins and a summary of their binding properties with *in vitro* translated p53. Numbers refer to amino acids.

Figure 6. (**A**) Schematic representation of the various E4F constructs used in colony formation assays and microinjection experiments. All the numbers refer to amino acids. (**B**) Expression pattern of the various E4F products. U2OS cells were transfected with 1 μg of plasmids expressing the indicated proteins or with empty vector (pcDNA3HA) and subjected to Western blot analysis with anti-HA antibody. (**C**) Subcellular distribution of the various E4F constructs represented in (**A**). Subconfluent U2OS cells were microinjected with vectors encoding the indicated proteins and processed for immunofluorescence staining with anti-HA antibody. The image was obtained by a Zeiss Axiovert 100 confocal microscope.

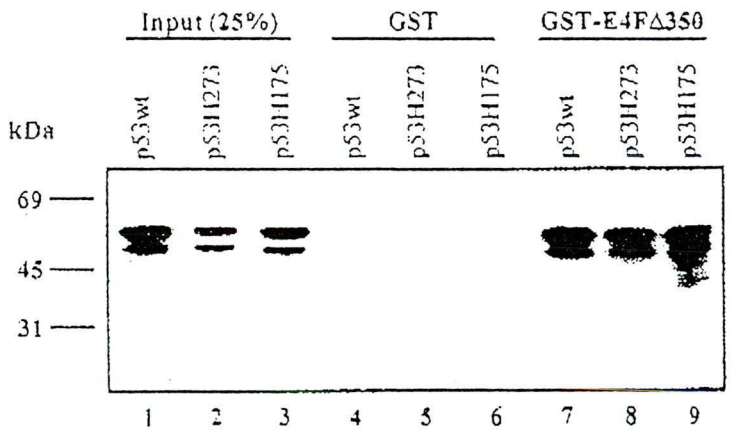
Figure 7. E4F inhibits S phase entry in a p53-dependent manner. (**A**) Colony formation assay in human cell lines. SaOS-2 and U2OS cells were transfected at low density with 1 μg of plasmid DNA and grown in the presence of 500 μg/ml G418 for two weeks. After selection the surviving colonies were fixed then stained with 10% Giemsa. The numbers represent the result of three independent experiments and calculated as compared to the number of colonies obtained by transfecting the empty vector (pcDNA3, 100%). (**B**) Growth inhibition assay by microinjection of E4F constructs in murine cell lines. NIH 3T3, Balb/c (10)1 and Val5 cells maintained at 37°C were coinjected with the indicated E4F expression vectors (50 ng/μl) and pGFP-C1 (25 ng/μl), used as marker. 20 hours after microinjection cells were pulsed with 50 μM bromodeoxyuridine (BrdU) for four hours, then fixed and stained with anti-BrdU antibody. The results are the mean of at least four independent experiments and were obtained by scoring more than 500 cells for each microinjected construct in every cell line. The percentage of S phase inhibition was calculated with the following formula: ((percentage of cells in S phase in the background - percentage of cells in S phase in the microinjected cells) / (percentage of cells in S phase in the background)) x 100. (**C**) Growth inhibition assay by coinjection of HA-E4F2.5K (50 ng/μl) or HA-E4F/PstI (50 ng/μl) and limiting amount (5 ng/μl) of wt p53 in Balb/c (10)1 cells. Inhibition of S phase entry was measured and calculated as described in (**B**). The data obtained from at least four independent experiments.
Error bars indicate standard deviation from the mean.

Fig. 1.

A


1
783
p120E4F
1
262
p50E4F
350
783
E4F△350
Zinc Finger Motif

B


Input (25%)
GST
GST-E4F△350
kDa
p53wt
p53H273
p53H175
69
45
31
1
2
3
4
5
6
7
8
9

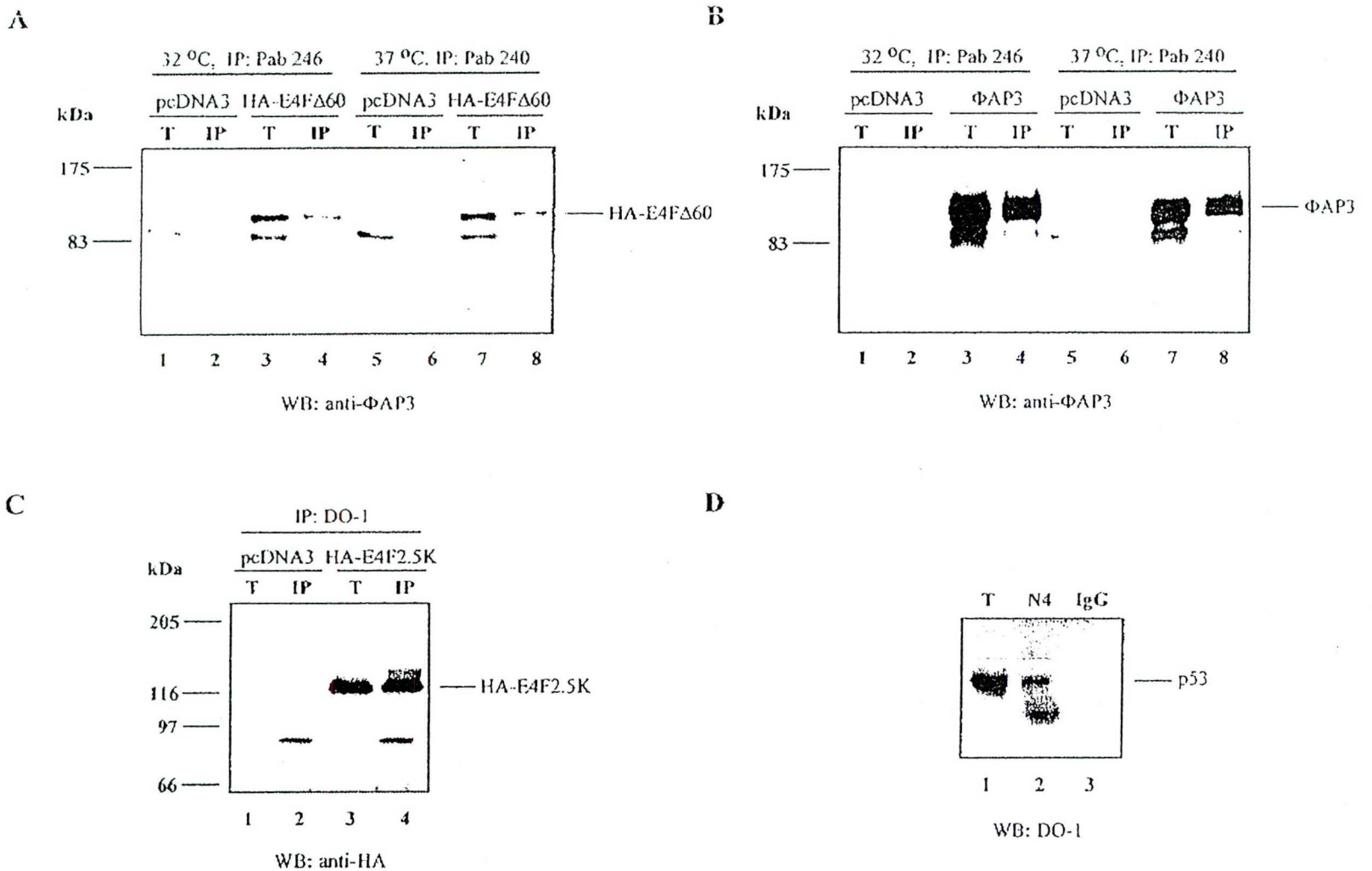
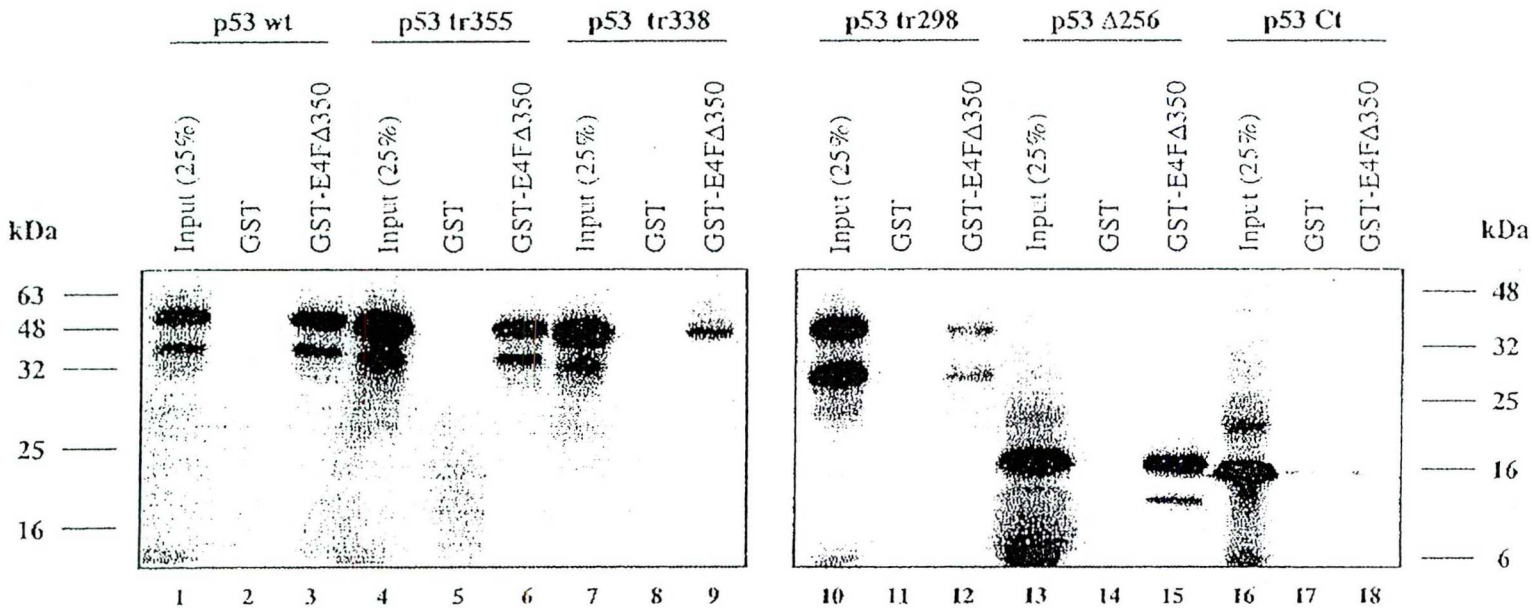

A
32 °C, IP: Pab 246
37 °C, IP: Pab 240
pcDNA3
HA-E4FΔ60
pcDNA3
HA-E4FΔ60
kDa
T IP T IP T IP T IP
175
83
HA-E4FΔ60
1 2 3 4 5 6 7 8
WB: anti-ΦAP3
B
32 °C, IP: Pab 246
37 °C, IP: Pab 240
pcDNA3
ΦAP3
pcDNA3
ΦAP3
kDa
T IP T IP T IP T IP
175
83
ΦAP3
1 2 3 4 5 6 7 8
WB: anti-ΦAP3
C
IP: DO-1
pcDNA3
HA-E4F2.5K
kDa
T IP T IP
205
116
97
66
HA-E4F2.5K
1 2 3 4
WB: anti-HA
D
T N4 IgG
p53
1 2 3
WB: DO-1

Fig. 2.

A


p53 wt
p53 tr355
p53 tr338
p53 tr298
p53 Δ256
p53 Ct
Input (25%)
GST
GST-E4FΔ350
kDa
63
48
32
25
16
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

B

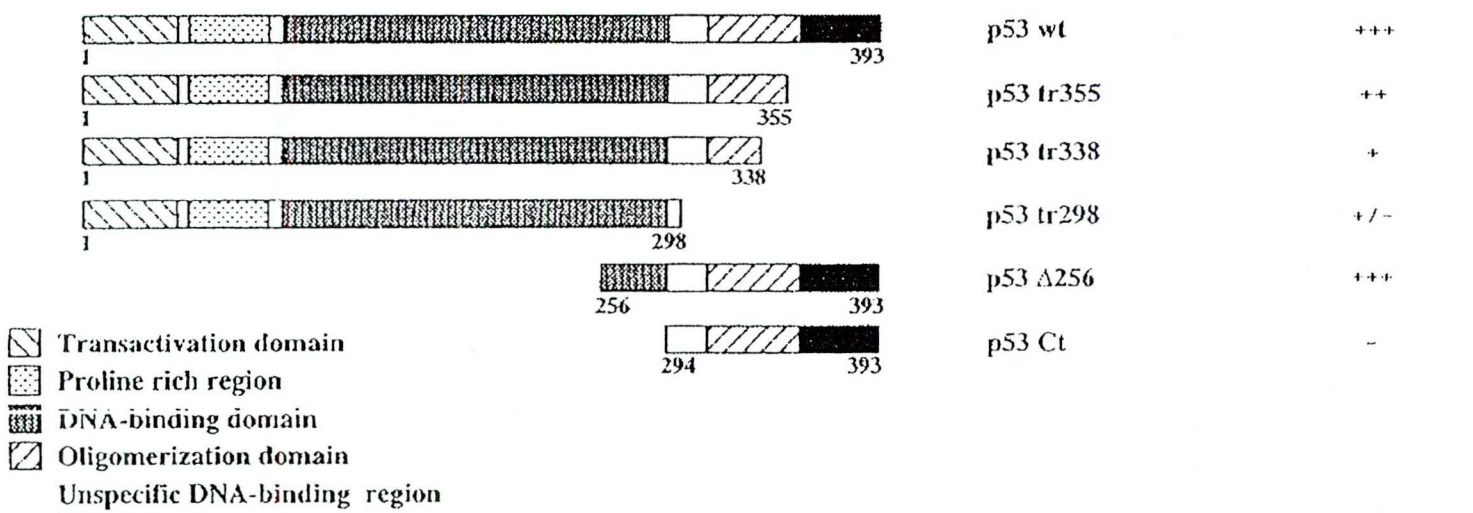
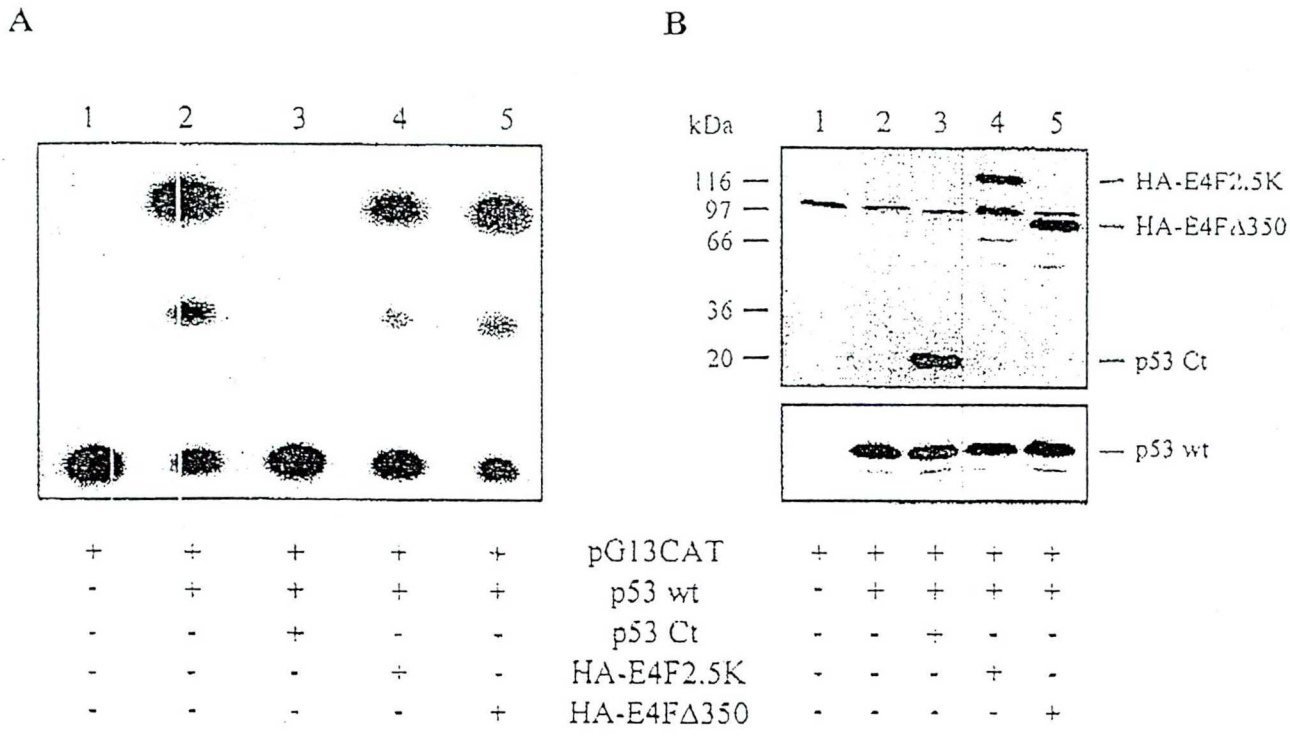

E4F BINDING
p53 wt +++
p53 tr355 ++
p53 tr338 +
p53 tr298 +/-
p53 Δ256 +++
p53 Ct -
1 393
1 355
1 338
1 298
256 393
294 393
Transactivation domain
Proline rich region
DNA-binding domain
Oligomerization domain
Unspecific DNA-binding region

Fig. 3.

Fig. 4.


A
B
1 2 3 4 5
kDa
116
97
66
36
20
HA-E4F2.5K
HA-E4FΔ350
p53 Ct
p53 wt
pG13CAT
p53 wt
p53 Ct
HA-E4F2.5K
HA-E4FΔ350

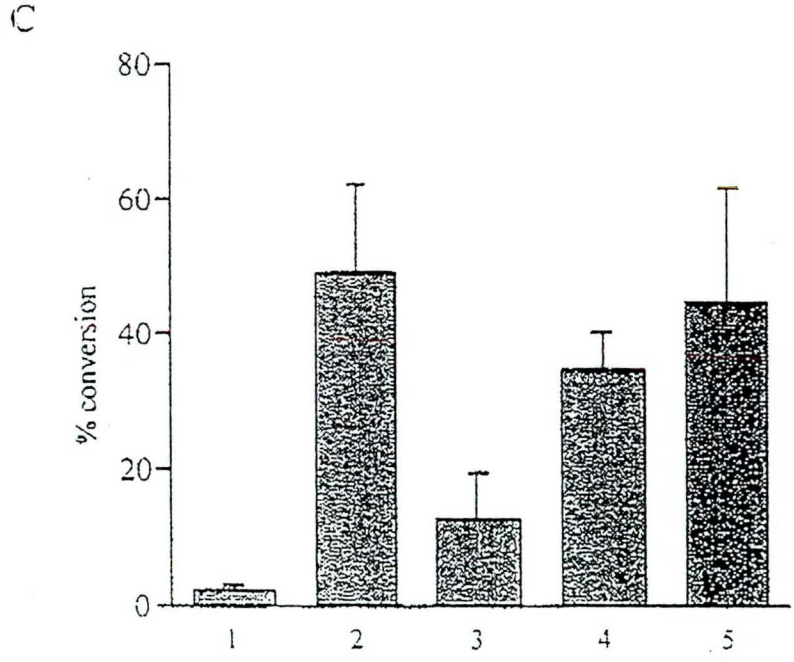
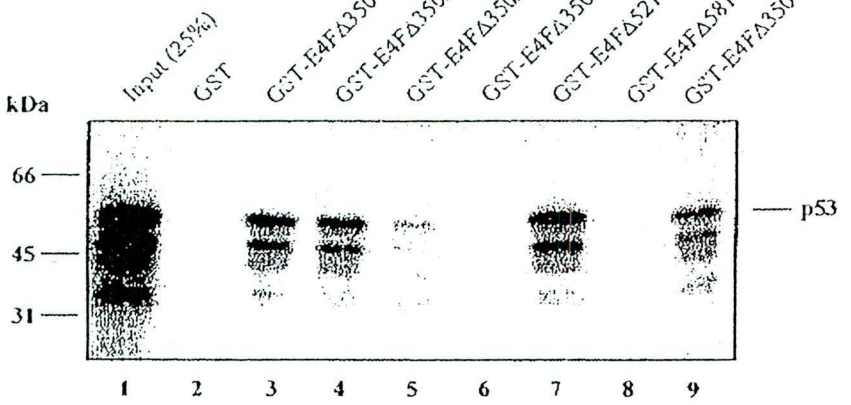
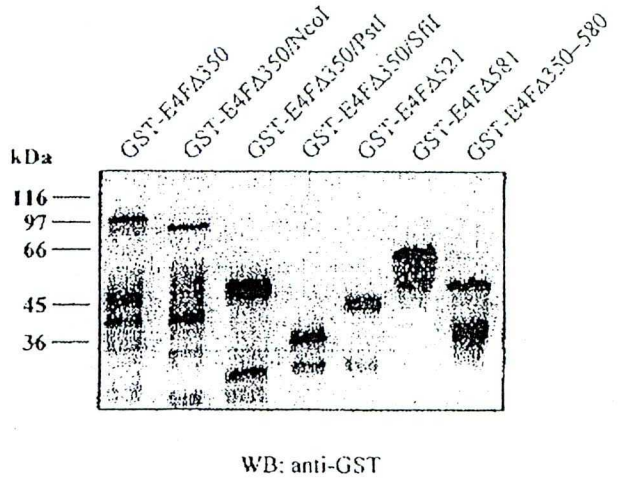

C
% conversion
80
60
40
20
0
1
2
3
4
5

Fig. 5.

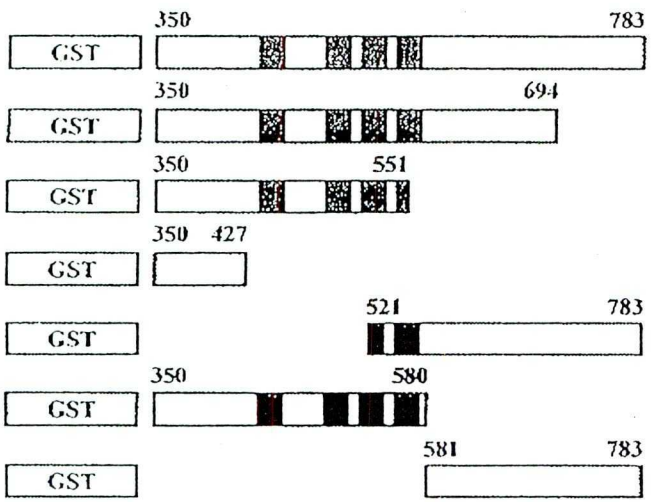
A



B



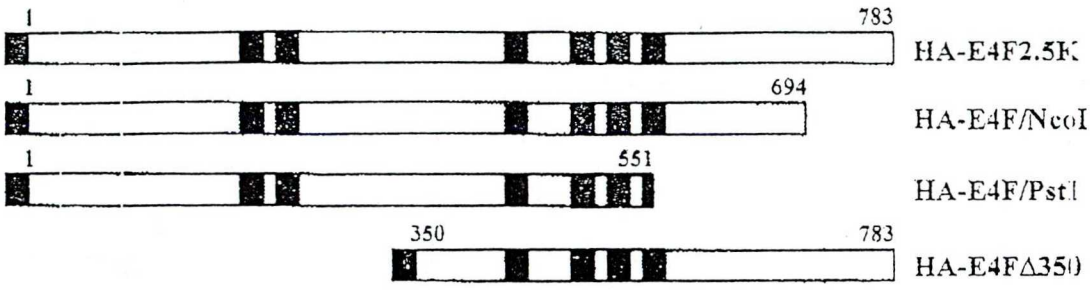
C



	p53 BINDING
GST-E4FΔ350	+
GST-E4FΔ350/NcoI	+
GST-E4FΔ350/PstI	+/–
GST-E4FΔ350/SfiI	–
GST-E4FΔ521	+
GST-E4FΔ350-580	+
GST-E4FΔ581	–

Fig. 6.

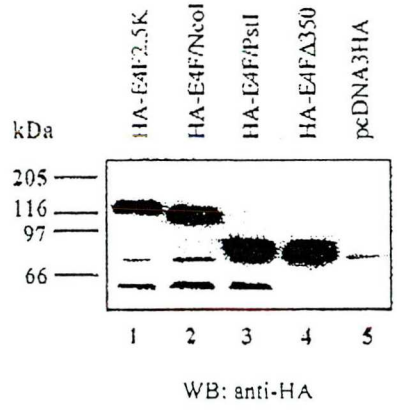
A



Zinc Finger Motif

HA-tag

B



WB: anti-HA

C

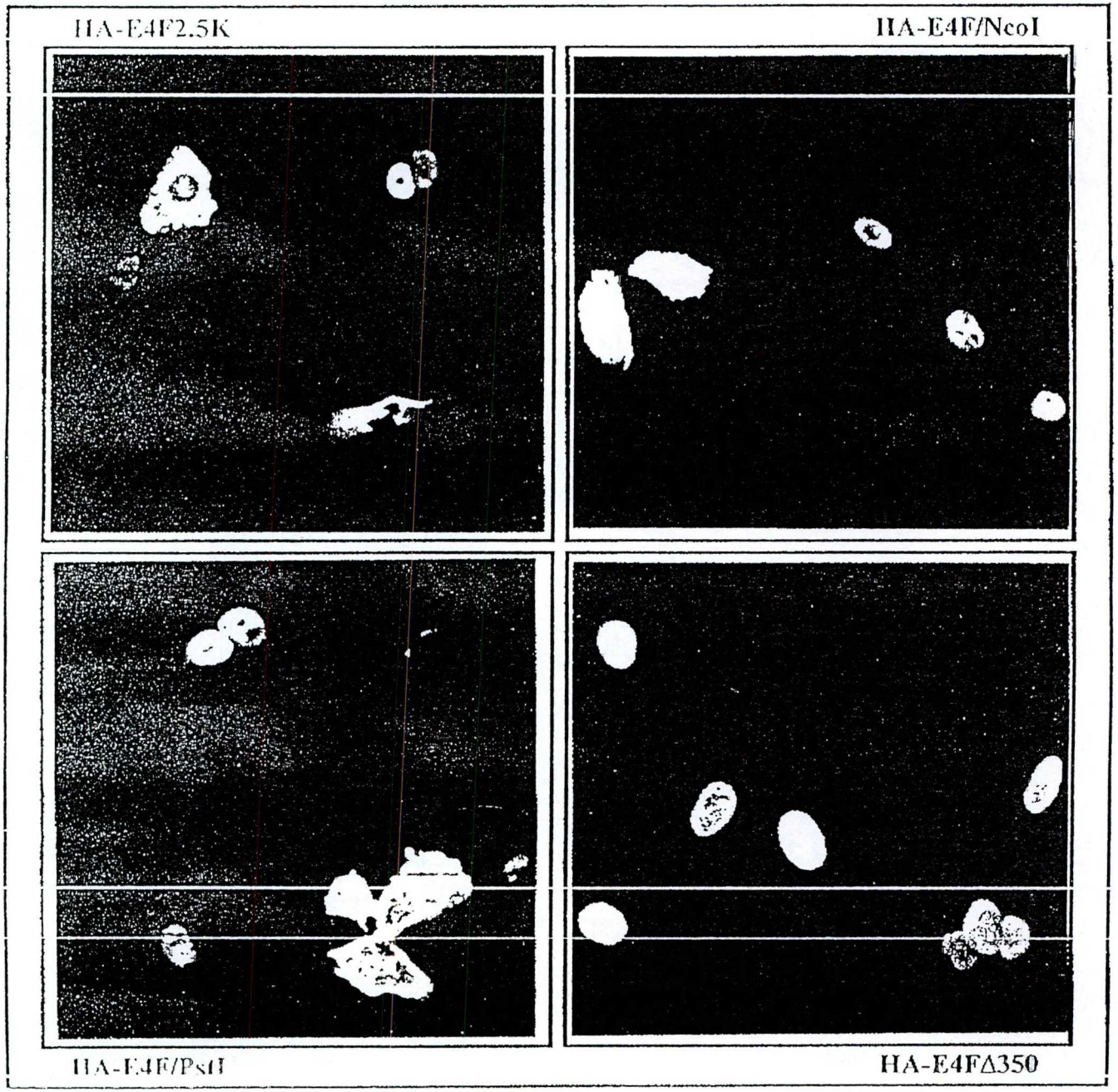
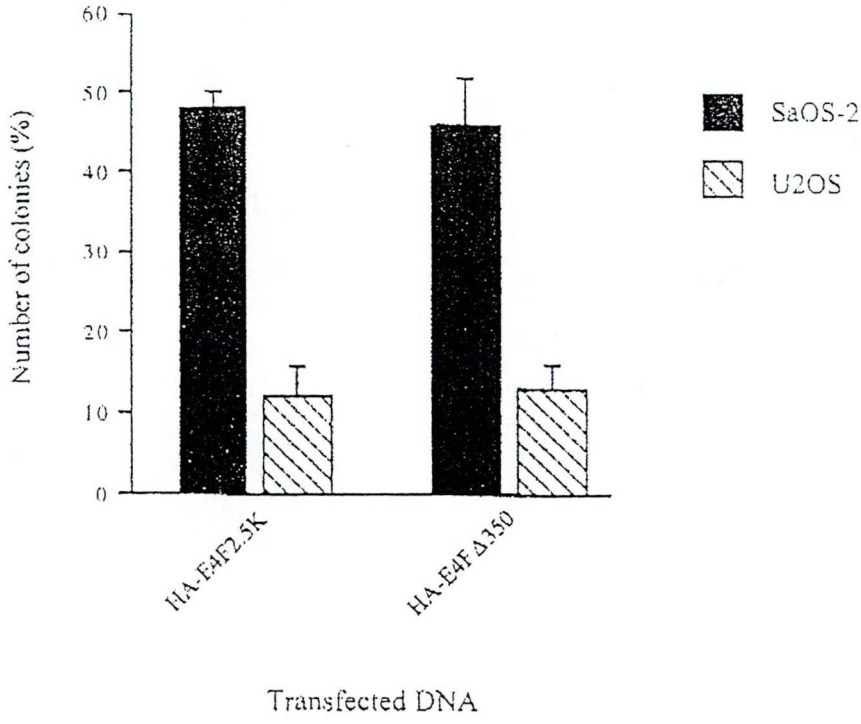


Fig. 6.

Fig. 7.

A


60
50
40
30
20
10
0
Number of colonies (%)
SaOS-2
U2OS
HA-E4F2.5K
HA-E4FΔ350
Transfected DNA

B

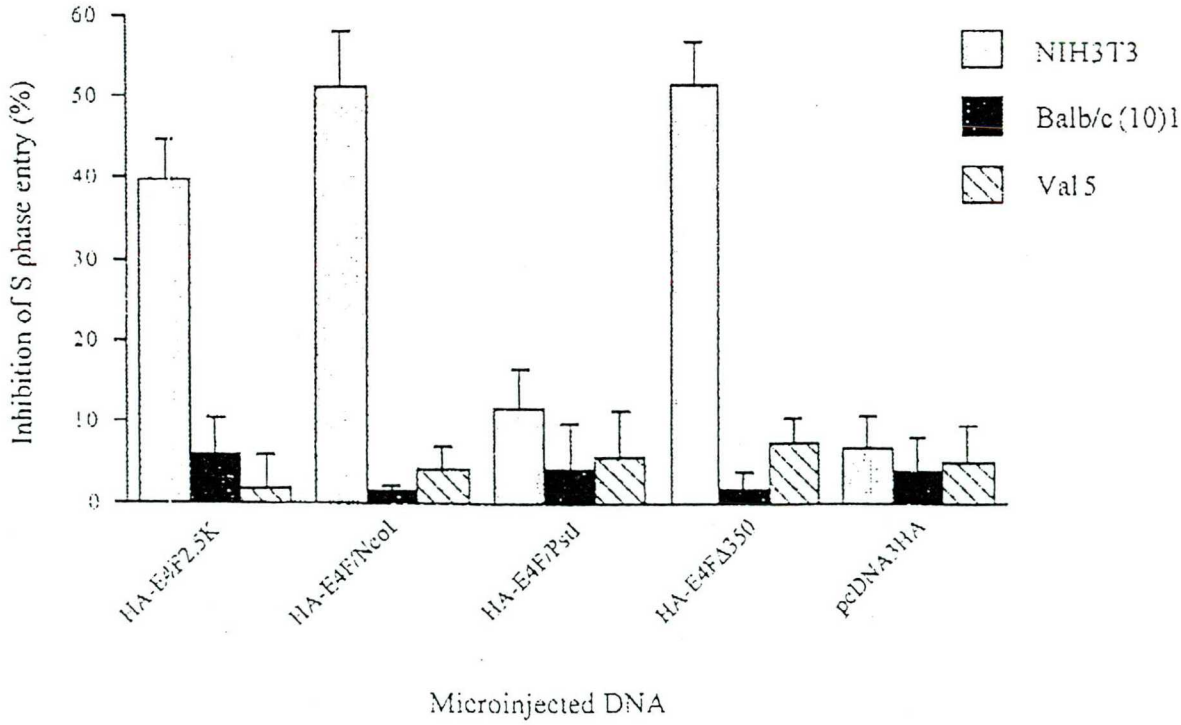
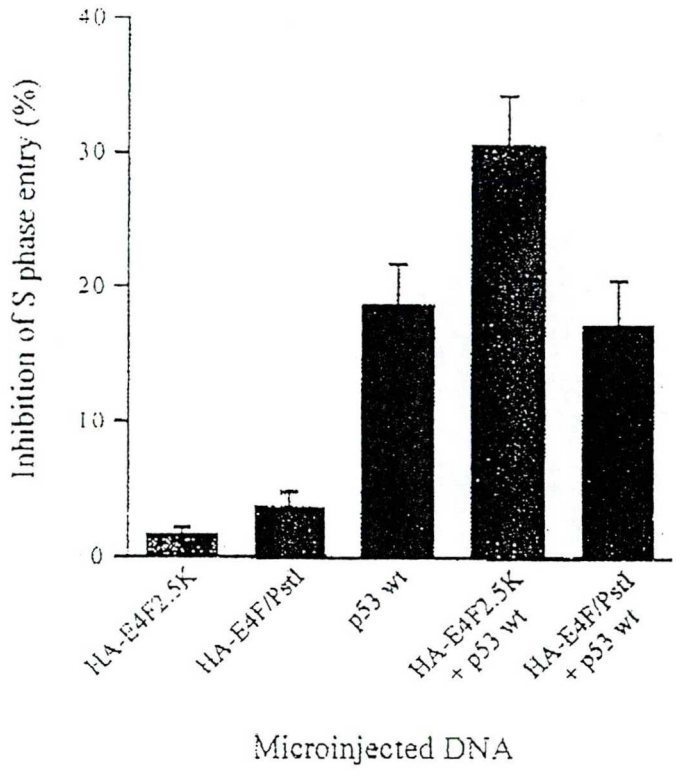

60
50
40
30
20
10
0
Inhibition of S phase entry (%)
NIH3T3
Balb/c (10)1
Val 5
HA-E4F2.5K
HA-E4F/Nco1
HA-E4F/Psu1
HA-E4FΔ350
pcDNA3HA
Microinjected DNA

Fig. 7.

C


40
30
20
10
0
Inhibition of S phase entry (%)
HA-E4F2.5K
HA-E4F/PstI
p53 wt
HA-E4F2.5K + p53 wt
HA-E4F/PstI + p53 wt
Microinjected DNA