The effects of tumor necrosis factor-alpha (TNF) on the differentiation and development of cultured myoblast and Sertoli cells

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Ph.D. thesis

1999

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Contents

Abbreviations

BSA	Bovine serum albumine
CAM	Cell adhesion molecule
Cdk	Cyclin dependent kinase
DMEM	Dulbecco's modified Eagle medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EDTA	Ethylene diamine tetraacetate
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
GM-CSF	Granulocyte-macrophage colony stimulating factor
HLH	Helix-loop-helix
IFN	Interferon
IL	Interleukin
LB	Luria-Bertani (medium)
LH	Luteinizing hormone
MHC	Myosin heavy chain
MLC	Myosin light chain
MRF	Myogenic regulatory factor
NK	Natural killer cell
PBS	Phosphate-buffered salt solution
RER	Rough endoplasmic reticulum
RVC	Ribonucleoside vanadyl complex
SDS	Sodium dodecyl sulfate
SCF	Stem cell factor or Steel factor
TBE	Tris-borate buffer
TGF	Transforming growth factor
TNF	Tumor necrosis factor-α
WT1	Wilms' tumor gene product

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Introduction

Most proteins are multifunctional. Were this not so, it is difficult to imagine that extraordinarily complex organisms could be fashioned through the selective expression of so few as 100,000 genes. As organisms became more complex and additional endocrine, paracrine, and autocrine mediators were needed, new functions were assumed by existing molecules. Such peptides may have similar or opposing functions and their functions may change with the state of differentiation. Another mechanism of diversification exists when a peptide is secreted from different cell types in response to different stimulators. These and other mechanisms greatly expand the informational potential of the genome in that a single gene may represent a unit that is eventually expressed as multiple signals that control a pattern of activity or behavior of a given organ. The cytokines are especially pleiotopic in their actions, and no cytokine is more pleiotropic than tumor necrosis factor-alpha (TNF).

TNF was originally characterized as a monokine that induces hemorrhagic necrosis of transplanted solid tumors in mice [16]. After cloning of the gene and purification of the recombinant protein, the effect of TNF upon many individual tissues and organ systems have been studied extensively and it soon became clear that the cytotoxic properties of TNF represent only one aspect of the functions of this protein and TNF also exerts biological effects on different normal cell types.

The best known function of TNF is the co-ordination 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. TNF can also behave as a growth factor and as a proliferation factor and it can induce a number of other growth factors, cytokines and their receptors.

The net biological effects of TNF vary according to tissue levels. At high concentrations it is detrimental to the host - in toxic shock and cancer cachexia - whereas at low concentrations its effects are beneficial - in tissue remodeling, inflammation and host defense. The beneficial effects of the TNF gene must outweigh the detrimental effects, since it has been conserved through evolution [1]. It was suggested that a possible reason for the conservation of the TNF gene throughout evolution is that it has an important role in

development [4]. It is possible that TNF has an indispensable function that is entirely distinct from any yet defined. Vlassara et al. [75] suggested that the ability of TNF to regulate cell death, growth, and proliferation indicates a role for it in normal tissue remodeling in the adult, and another possible reason for the conservation of the TNF gene, despite the risk associated with its presence in the genome, is that it has a fundamental role in tissue remodelling and morphogenesis during development of the embryo.

Although cytotoxic effects of TNF on tumor cell lines of many species have been known for more than 20 years, only recently has a role for TNF in normal cellular function been identified.

TNF is produced predominantly by activated macrophages, but it can also be produced by activated T and B lymphocytes, NK cells, Kuppfer cells, tumor cell lines, smooth muscle cells, activated keratinocytes, glial cells, adipocytes and a number of other cell types. TNF is a 17-kDa peptide. Human TNF is translated as a 233 amino acid precursor containing a hydrophobic sequence that is proteolytically cleaved to the mature 157 amino acid form (17 kDa). The biologically active secreted form of TNF is a noncovalently bound trimer. A cell membrane-associated form of TNF (26 kDa) also possesses bioactivity and is proteolitically cleaved to release the active secreted moiety (17 kDa) and a smaller membrane-associated fragment.

TNF binds to specific receptors displayed upon the cells of virtually every tissue, and effects a vast array of changes in cell behavior, many of which impact upon homeostasis of the organism as a whole. The trimeric TNF exerts its effect by binding to and clustering specific high-affinity receptors which are present on the plasma membrane of almost all cell lines, the number varying between 100 and 10,000 [25]. So far, two TNF receptors with a molecular mass of 55 kDa (p55) and 75 kDa (p75) have been identified and cloned that are independently expressed [41]. Although the extracellular domains of the two TNF receptors are 28% identical, no homology has been found between the intracellular domains of the two proteins [21]. This observation suggests that the two receptors may be responsible for inducing distinct cellular responses. TNF exhibits a wide variety of biological actions, but which receptor is responsible for these biological actions is not well characterized.

One of the key questions in developmental biology is understanding the molecular mechanisms that regulate gene expression during tissue differentiation. This differentiation process requires the coordinate expression of many different genes. Although genes coding for several tissue-specific proteins have been isolated, the regulatory mechanisms underlying their coordinated expression are still poorly understood.

The process of morphogenesis and cell differentiation are controlled by soluble factors and by the microenvironment. A range of inflammatory cytokines has been detected at early stages of development. Rothstein et al. [65] have constructed cDNA libraries from various stage of pre-implantation mouse embryos and have detected transcripts of IL-6, IL-1β, IFN-γ, IL-3, and GM-CSF. These workers point out that these cytokines not only regulate proliferation, but also induce differentiation. This may also true of TNF, which is a member of system of cytokines that induces the above-mentioned factors.

The presence of TNF and its membrane bound precursor form was detected during fetal and neonatal development of the mice, both in the embryos [56] and in the culture fluids during *in vitro* fertilization [78]. Mouse blastocysts express the 55 kDa receptor of TNF [M26] and both receptor types have been identified in mouse embryos [27, 36]. Gendron et al. [26] reported upon the expression of high-molecular-weight proteins cross-reactive with TNF antibodies in the developing nervous systems of mouse and chick embryos. Jaskoll et al. [35] prooved that the 17-kDa TNF and the 55-kDa TNF receptor are expressed in developing mouse lungs and that TNF enhances lung branching morphogenesis *in vitro*.

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 [38]. 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.

Yamasu et al. [84] have proposed a model for the role of TNF in development known as „ontogenic inflammation", which is based on the polypeptide mediator network, present during inflammation. Wride and Sanders [79] proposed that TNF may have three distinct roles during embryonic development, analogous to its roles in the immune system and during inflammation. These roles include effects on programmed cell death; on cellular growth and

differentiation; and on the remodelling of the extracellular matrix (ECM) and the regulation of the synthesis of cell adhesion molecules (CAMs) and integrins.

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. Despite the studies revealing the presence of TNF and other inflammatory cytokines in developing embryos, virtually nothing is known of their specific functions. Speculation on a role for cytokines in development has usually been based on studies of the interaction of the immune and reproductive systems during pregnancy.

Proper development of a multicellular organism is extremely complex, the realization of the program requires precise spatial and temporal control of cell proliferation and co-ordinate 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.

Studying the effects of TNF on different cell lines and their viral oncogene transformed derivatives we noticed morphological changes caused by TNF. In preliminary experiments we have shown that TNF preserved the pluripotence of embryonic stem cells and inhibited the growth of trophoblast cells and induced a dedifferentiation-like process in embryonic chondrocytes [71]. These data suggest a possible role for TNF in normal development.

Skeletal muscle myoblast cells in culture are useful model for studying cell differentiation. Muscle cell differentiation is marked by myoblast proliferation followed by progressive fusion to form large multinucleated myotubes that synthesize muscle-specific proteins and contract spontaneously. 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. The fusion of mononucleate myoblast cells into multinucleate myotubes (myogenesis) has often been studied as a model system of terminal cellular differentiation.

Regeneration of mature skeletal muscle after injury recapitulates fetal myogenesis. It is initiated by activation of the reverse myogenic precursor cells, the satellite cells. Each of the developmental steps is characterized by the transcriptional activation of specific sets of genes,

most notably during terminal differentiation when the muscle isoforms of contractile proteins are expressed, and affected by cell interactions with ECM [46].

The MyoD family of transcription factors participates in the regulation of the complex phenomenon of myogenic differentiation during development and *in vitro*. These myogenic regulatory factor (MRF) genes are expressed only in skeletal muscles and its embryonic precursor cells. This family of proteins, which includes MyoD [19], myogenin [80], Myf-5 [7], and MRF4 [64] in mouse (and Myf-3, Myf-4, Myf-5, Myf-6 in human, respectively) regulate the entire family of muscle-specific proteins. It has been shown that each member of the MyoD family activates not only genes encoding muscle-specific proteins but they also transactivate each other [72]. The MRF proteins contain a conserved basic DNA binding domain. The myogenic basic helix-loop-helix (bHLH) proteins bind to DNA in the form of heterodimers with ubiquitously expressed bHLH factors termed 'E proteins' [39]. Binding sites for myogenic bHLH-E protein heterodimers, which share the consensus nucleotide sequence CANNTG (termed the E box), are necessary for the expression of several skeletal muscle-specific genes, such as muscle creatine kinase, myosin light-chains (MLCs) 1 and 3, desmin, acetylcholinesterase and the acetylcholine receptor. The activity of the MRFs is regulated at the post-translational level by protein-protein interactions and phosphorylation. 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 [13, 76]. 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 [57].

As determined by *in situ* hybridization, MRF transcription occurs sequentially during the murine skeletal muscle developmental program. The seemingly complex expression patterns of mRNAs observed by *in situ* hybridization are probably the consequense of succesive waves of myogenic differentiation. Hierarchical relationships and functional redundancy among the MRF genes was revealed by gene targeting. Mice lacking MyoD are viable and have seemingly normal skeletal muscle [66]. Newborn mice lacking Myf-5 also have apparently normal skeletal muscle, but die perinataly due to rib deformities [8]. Mice lacking myogenin are immobile at birth and contain myoblasts that fail to differentiate efficiently into myotubes, however, normal numbers of myoblasts were present and these were organized in groups similar to wild-type skeletal muscle, suggesting that myogenin is not

involved in patterning [54]. Mice lacking both Myf-5 and MyoD display a complete absence of skeletal myoblast and myofibers [67]. The fourth myogenic factor, MRF4, may function late in the myogenic pathway, as suggested by its expression pattern.

Cultured myoblast cell lines express Myf-5 mRNA and/or MyoD mRNA 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 [30]. Commitment to myogenesis is facilitated by autoactivation of the MyoD promoter by MyoD protein [72]. In proliferating myoblasts, myogenin fails to be expressed and its synthesis starts only at the onset of differentiation [6], upon myotube fusion, and MRF4 mRNA several days after fusion [49]. Serum components, basic FGF, TGF-β and EGF, agents that suppress differentiation of muscle cells *in vitro*, down-regulate the activity of the myogenin gene [10, 32]. There is a convergence of growth factor-signalling pathways and pathways controlled by members of the MyoD family and the decision of myoblasts to differentiate or devide is dictated by a ballance between these antagonistic programs.

Skeletal muscle originates in vertebrates from a small pool of progenitor cells that arise in the early somite and precordal mesoderm as a consequence of inductive signals received from the neural/notochord complex [14]. How these signals activate MRF transcription is not at all clear and this is now an active area of study. It is unclear at this time how these myogenic regulatory genes themeselves are controlled during myogenesis.

We have also investigated 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 [23],including their hormonal control. Moreover, this cell type is implicated in the immune tolerance of testicular autoantigens, both by establishing the blood-testis barrier, which segregates autoantigenic germ cells, and by secreting immunosuppressive factors into the interstitial environment. The production of mature spermatozoa requires a complex interaction between Sertoli cells and germ cells. Sertoli cells regulate aspects of germ cell division and differentiation while germ cells provide signals that modulate Sertoli cell functions. There are probably a number of growth factors and cytokines that both stimulate and regulate the many differentiation steps in this complex processes, which generally requires 1-2 months to complete in most mammals.

Sertoli cell precursors, which are the first cell type of the testis, form in the mouse embryo between 10.5 and 11.5 days *post coitum*, in response to the expression of a Y-chromosome encoded transcriptional regulator, the testis determining factor (Sry) [37]. During embryogenesis, these cells coalesce to form the precursors of the adult seminiferous tubules, the sex cords, which come to enclose the male germ cells, preventing their entry into meiosis and arresting mitotic activity in the germline. Before puberty, Sertoli cell division stops, testicular size is fixed as each Sertoli cell supports a fixed number of germ cells. Throughout spermiogenesis, differentiating spermatids are supported by Sertoli cells and form highly ordered, characteristic cellular associations within the seminiferous tubule. The three-dimensional structure of the seminiferous tubule is essential for creating the environment required for germ cell differentiation. Cell adhesion molecules (CAMs) play a role in developmental events and maintenance of tissue architecture and may increase adhesive contact between Sertoli and germ cells [24].

Evidence is accumulating that paracrine and autocrine factors from Sertoli and germ cells are important in the functioning of both cell types [28]. 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. The appearance of Sertoli-Sertoli junctional complexes occurs in most mammals at about the same time as the start of meiosis by germ cells. This barrier formation may have a role in the onset of spermatogenesis. The factors responsible for stimulating formation of junctional complexes between adjacent Sertoli cells have not been conclusively identified. Testicular cell-to-cell communication in the testis might be exerted by growth factors and cytokines. A potential source of cytokines within the testis are the macrophages which represent about 20% of testicular interstitial tissue [47]. Sertoli cell tigh junctions exclude molecules exceeding approximately 10 kDa and probably exclude TNF (17 kDa) from the adluminal compartment, but in the testis, TNF is known to be produced also by germ cells (round spermatids) [20] and to affect Sertoli cell activity by binding to the p55 receptor [44]. TNF binding sites per Sertoli cells is 1300 [43]. The presence of the TNF binding sites on Sertoli cells are consistent with the findings of Veldhuis et al. [74], who demonstrated the presence of TNF receptors on granulosa cells, the female homologue of Sertoli cells. Spermatids release TNF, which is detected by Sertoli cells (p55) and may serve as a paracrine factor, regulating an as yet unidentified processes in

spermatogenesis as suggested by De et al. [20]. As TNF expression was detected in spermatogenic cells and Mohari et al. [51] have shown the presence of the transmembrane form of TNF on mouse testicular germ cells 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 [60]. 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 [63]. 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 and they are capable of phagocytosis.

Tumor suppressor genes and oncogenes that control proliferation and apoptosis are also known to play an important role in development and embryogenesis. 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. In searching for new p53 interacting proteins, we isolated the transcription factor p120E4F in a yeast two-hybrid screen. We demonstrated that E4F associates both *in vitro* and *in vivo* with p53 in mouse and in human cells, and the association is required for its growth suppression activity. P120E4F may therefore represent a novel p53 cellular partner that contributes to its checkpoint functions. (Details see in Annex III.)

Matherials and methods

1. Cell culture

C2 mouse myoblast cell line [83] 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 supplemented with 10% fetal calf serum (Inst. Jacques Boy, France), 100 U/ml penicillin G and 100 μg/ml streptomycin, at 37°C. Cell cultures were split every 2 or 3 days.

45T-1 cells, expressing Sertoli cell markers, derived from testicular tumor tissue of a mouse expressing the polyoma large T antigen in its gonads [60], were cultured in DMEM containing 10 % FCS 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.

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 plasmamembrane 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 5 % serum.

2. 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 by a combination of ion exchange, reverse phase and affinity chromatographies in our laboratory. 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.

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

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) 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 x 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) and immobilized TNF columns. 10 µl of the dialyzed, sterile filtered preparation protected L929 or WEHI cells against the cytotoxic effects of 1 µg (>2 x 10^4 U) TNF.

3. In vitro TNF cytotoxicity assay

Bioassay of TNF was based on its cytotoxicity, measured on mouse L929 tumor cells. L929 cells were grown in DMEM with 10% serum. 100 µl of cell suspension ($3x10^4$ cells/well) was mixed with 100 µl of serial dilutions of TNF in 96-well flat-bottom culture plates in the presence of 5% serum and 1 µg/ml actinomycin-D. After 18 hours of culture at 37 °C, viability of the cells were determined by the tetrazolium salt (MTT) assay: 100 µl of MTT [3-(4-5-dimethythiozol-2-yl)2-5-diphenyl-tetrazolium bromide] (SIGMA) solution (5 mg/ml) in PBS was added to each well. After 2-h incubation at 37 °C the medium was removed and the converted dye was solubilized with 100 µl acidic isopropanol (0.05 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 540 nm with background substraction at 690 nm. The amount of TNF required to mediate the half-maximal cytotoxicity on L929 cells was assigned a value of 1 unit.

4. Rabbit immunization

Rabbits were immunized by intradermal injection of 5 x 10^6 purified recombinant human TNF-α in incomplete Freund's adjuvant denatured by 2-min boiling. The treatment was repeated weekly. The neutralizing activity of the antisera was tested on L929 cells.

5. Titration of the neutralizing capacity of immunized rabbit sera

Different amounts of the rabbit serum (1-10 µl) were added to serially diluted standard TNF into DMEM containing 5 % FCS, 1 µg/ml actinomycin D, in 96-well plates. After 30 min incubation at 37 °C, the samples containing the TNF-anti-TNF complexes were added to L929 cells, grown in 96-well plates. (Conditions see at the cytotoxicity assay.) Following a 16 hours incubation at 37 °C , the cell viability and the TNF counteracting capacity of the samples were determined.

6. [^{3}H]-thymidine incorporation assay

The proliferation of myoblasts and Sertoli cells 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 labelled with 10 µCi/ml [^{3}H]-thymidine (25 Ci/mmol, Amersham) in a volume of 0.25 ml/well of culture medium for 2 hours 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.

7. 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: $pMLC1_F$ [18].

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 [77].

3. The probe for the mouse skeletal α-actin ($\alpha_{SK\text{-}actin}$) was isolated from a pBR322 derivative, pAM91-1 [50].

4. pEMCIIs was used as a probe for the mouse myogenic regulatory factor, MyoD. *MyoD* gene was inserted into pEMSV [19].

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 to Bluescript [7]. All these plasmids were kindly provided by Prof. P. Amati (Univ. di Rome La Sapienza, Rome, Italy).

	Plasmid name	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

Table 1. Molecular probes used in these studies
Ap: Ampicillin, Tc: Tetracycline

8. 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 4,000 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 4,000 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 hour of incubation at 37°C, 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.

9. 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 13,000 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)). After boiling for 1 min and centrifugation for 10 min at 13,000 rpm, plasmid DNA was precipitated from the supernatant by 0.2 ml of cold isopropanol at room temperature. After centrifugation at 13,000 rpm at 4°C for 10 min, DNA was dissolved in distilled water.

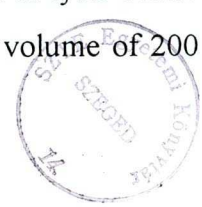
10. Electroelution

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 hour electrophoresis the buffer surrounding the gel slice was recovered and the DNA was precipitated with 0.1 volume of sodium acetate (pH5.2) and 2 volumes of 96% ethanol, overnight at -20 °C After centrifugation (13000 rpm, 15 minutes) 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 Table 1.).

11. Isolation of RNA

RNA samples were isolated from cultured myoblast cells by using the ribonucleoside vanadyl complexes (RVC) method. Cells (5-10 x 10^6) were harvested from confluent cultures by trypsinization (cell aggregates were disrupted by sonication), 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/ml RVC (Sigma) was



added. After centrifugation at 13,000 rpm at 4 °C, the supernatant was mixed with equal volumes of extraction buffer (0.2 M Tris-HCl, pH 8.0, 0.35 M NaCl, 20 mM EDTA, 1% SDS), a mixture of chloroform and isoamyl alcohol (49:1) and Tris-EDTA saturated phenol. After brief shaking the mixture was separated by centrifugation at 13,000 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.

12. Northern blotting

Total RNA (20 μg/lane) was electrophoresed in 1.25 % agarose/formaldehyde gel, and transferred to a nylon membrane (Hybond-N, Amersham) with 20 x 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 spectrophotometer at 260 and 280 nm.

Prehybridization was performed in 0.25 M Na_2HPO_4, 7% SDS (pH 7.2) and 250 μg/ml sonicated chicken blood DNA, at 65°C for 1 hour. Hybridization was performed overnight in the same buffer containing 10^6 cpm/ml of [^{32}P]- cDNA probes, labelled by random primer synthesis with [^{32}P]-dCTP (Izotóp Intézet Kft.) using the Oligolabeling kit (Pharmacia), 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-phospate dehydrogenase (PGAD) probe (pHcGAP, from ATCC, USA) was used to determine the approximate RNA contents in the respective lanes.

13. Preparation and staining of sections of cell aggregates

C2/7 myoblast and Sertoli 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.) 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.

14. Immunohistochemistry

Cells grown on glass coverslips were 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) for 60 min. Antibodies against mouse desmosomal protein, vinculin, cytokeratin CK5, vimentin and laminin (all from Sigma) and anti-alpha and beta tubulin and anti-cytoskeletal actin (both from Amersham) were applied on the coverslips in dilutions according to manufactural instructions (prepared in PBS, containing 0.2 % BSA). 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) 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 (Carl Zeiss).

15. Electron microscopy of myoblast cells

For electron microscopy C2/7 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.

16. Transmission and scanning electron microscopy of Sertoli cells

For electron microscopic investigations samples from each experimental groups were fixed in 2.5 % glutaraldehyde containing 1 % sucrose and 2 mM $CaCl_2$, buffered by 100 mM cacodylate, pH 7.0, for 1 hr at room temperature. After washing in cacodylate buffer they were postfixed 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). Ultrathin sections were stained by lead citrate and examined in a JEOL CXII electron microscope.

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 hr 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 scanning electron microscope.

Results

1. Effects of TNF on C2/7 myoblast cells

1.1. Both soluble and transmembrane form of 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 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 (10^5 NU/ml) 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 (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 expression 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 plasmamembrane 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).

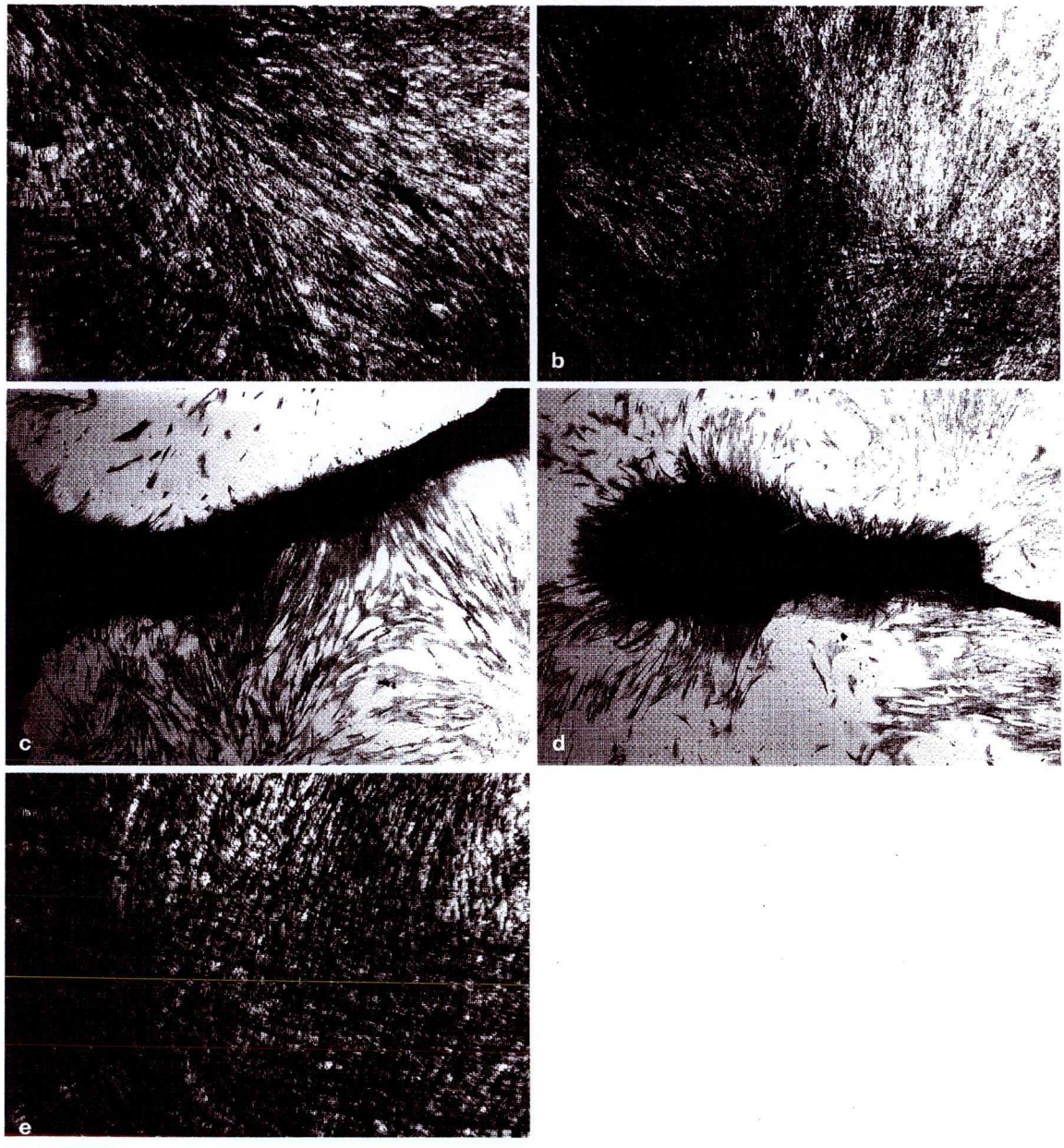


Fig. 1. Effect of TNF on the cells of myoblast monolayers.

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 the aggregation. Control monolayer is shown on (**e**) on the fifth day of culture.

1.2. 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 (Table 2.) 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 15 ng/ml	TNF 50 ng/ml	TNF 150 ng/ml
1. day	158+/-31	180+/-18	140+/-22
2. day	128+/-17	117+/-26	115+/-22
3. day	83+/-14	78+/-22	67+/-19
10. day	56+/-12	65+/-20	34+/-8

Table 2. The effect of TNF treatment on the proliferation of myoblasts. 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.

1.3. 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 [6], 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 it can be seen on Fig. 2., TNF exerted a significant 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.

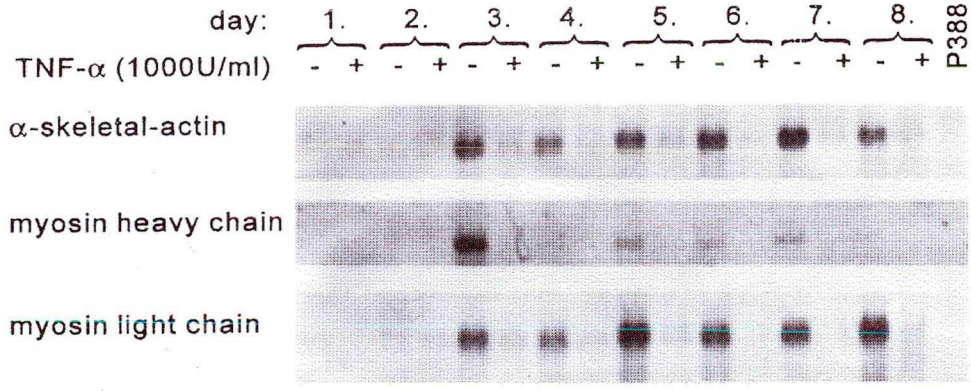


Fig. 2. TNF inhibited the expression of muscle specific genes.
Expression of the genes of myofilament components in myoblast cells decreased in TNF-treated cells compared to that of controls. Myoblast cells were cultured on 24-well plates (10^5 cells/well). Fresh medium with or without 50 ng/ml TNF was 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. RNA extacted from P388 mouse myeloid leukemia cells served as negative control.

1.4. Organized myofilaments are formed only in control myoblast cells.

TNF treated cells, probably as a result of the low level expression of the myofibrillar proteins, contained no organized filaments (Fig. 3.).

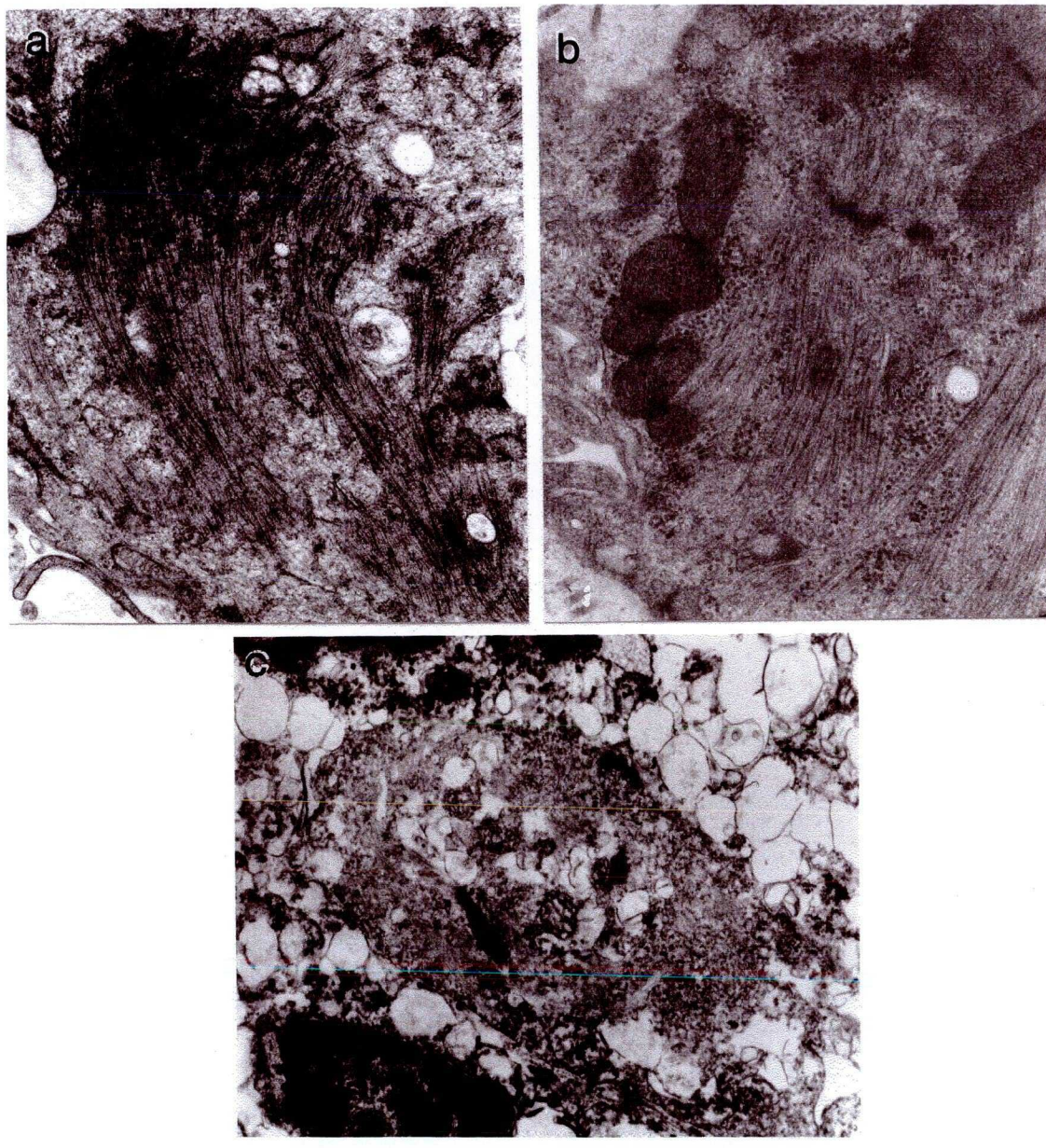


Fig. 3. Electron microscopic picture of a 10 days old C2/7 myoblast culture (**a**) and a human muscle biopsy (**b**) shows the presence of organized myofilaments, the sign of normal myogenic differentiation. In the cells of the aggregates of TNF-treated myoblast cells (**c**) similar structures can not be observed. The culture shown on **c** was exposed to TNF for 10 days (40,000 x magnification)

1.5. 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 Fig. 4. shows, TNF treatment inhibited the accumulation of transcripts of myogenic regulatory factors in C2/7 mouse myoblasts.

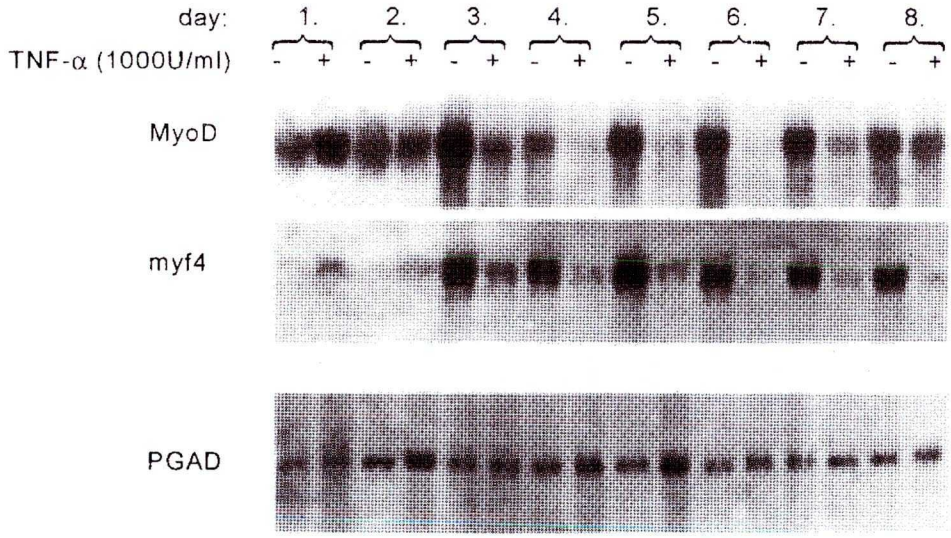


Fig. 4. The effect of TNF on the synthesis of transcripts of myogenic regulatory factors. Northern analysis of total RNA extracted from TNF treated (50 ng/ml) and non-treated cells. Expression of myogenic regulatory factors MyoD and myogenin was strongly inhibited in TNF treated cells. Transcripts of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

1.6. Myoblast cell aggregates contain collagen fibres

We observed a significant difference between collagen fibre content of TNF treated and control cells. Collagen fibres can be seen in a number of electron microscopic pictures of TNF induced-myoblast cell aggregates (Fig. 5.). The rate of collagen synthesis is increased during the cell to cell contact step of myogenesis [55] and according to literary data prefusion myoblast synthesize type I, III and V collagen [3, 68]. We could not detect any visible sign of collagen accumulation in the control cells undergoing normal myogenic differentiation.

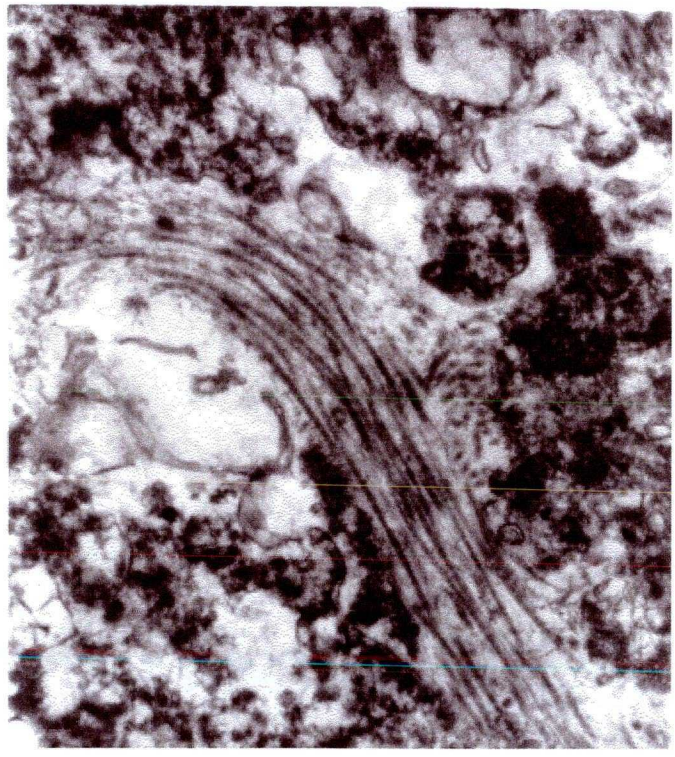


Fig. 5. Collagen fibers in TNF-treated myoblast cells.

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 x magnification).

1.7. TNF does not trigger the synthesis and release of TGF-beta

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 mRNS 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 the same level of TGF-β messages (not shown).

2. Effects of TNF on 45T-1 Sertoli cells

2.1 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 to10^4 U/ml) TNF at 32 °C for 6-24 hr 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 dinamic formations and 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. 6.).

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, Sigma) was maintained, though at a reduced level (data not shown).

Microscopic investigation of intact aggregates showed tubular or rod-like inner structures. Frozen sections revealed highly ordered organizations of the cells into garland-like formations (Fig. 7.a) that highly resembles to seminiferous tubules of testis. No aggregation or differentiation was observed in control cultures of 45T-1 cells in the absence of TNF. Control 45T-1 cells grew into multilayered cultures if kept for long periods of time. These formations however, are flat and show no differentiation or inner organization in cross sections (Fig. 7.c).

In well developed spheroids three structural domains could be distinguished by light (Fig. 7.a) and electron microscopy (Fig. 7.b): *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 prominent cortical network of microfilaments, ovoid nuclei, well developed RER and numerous cells contacts and desmosomes (Fig. 8.a-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.

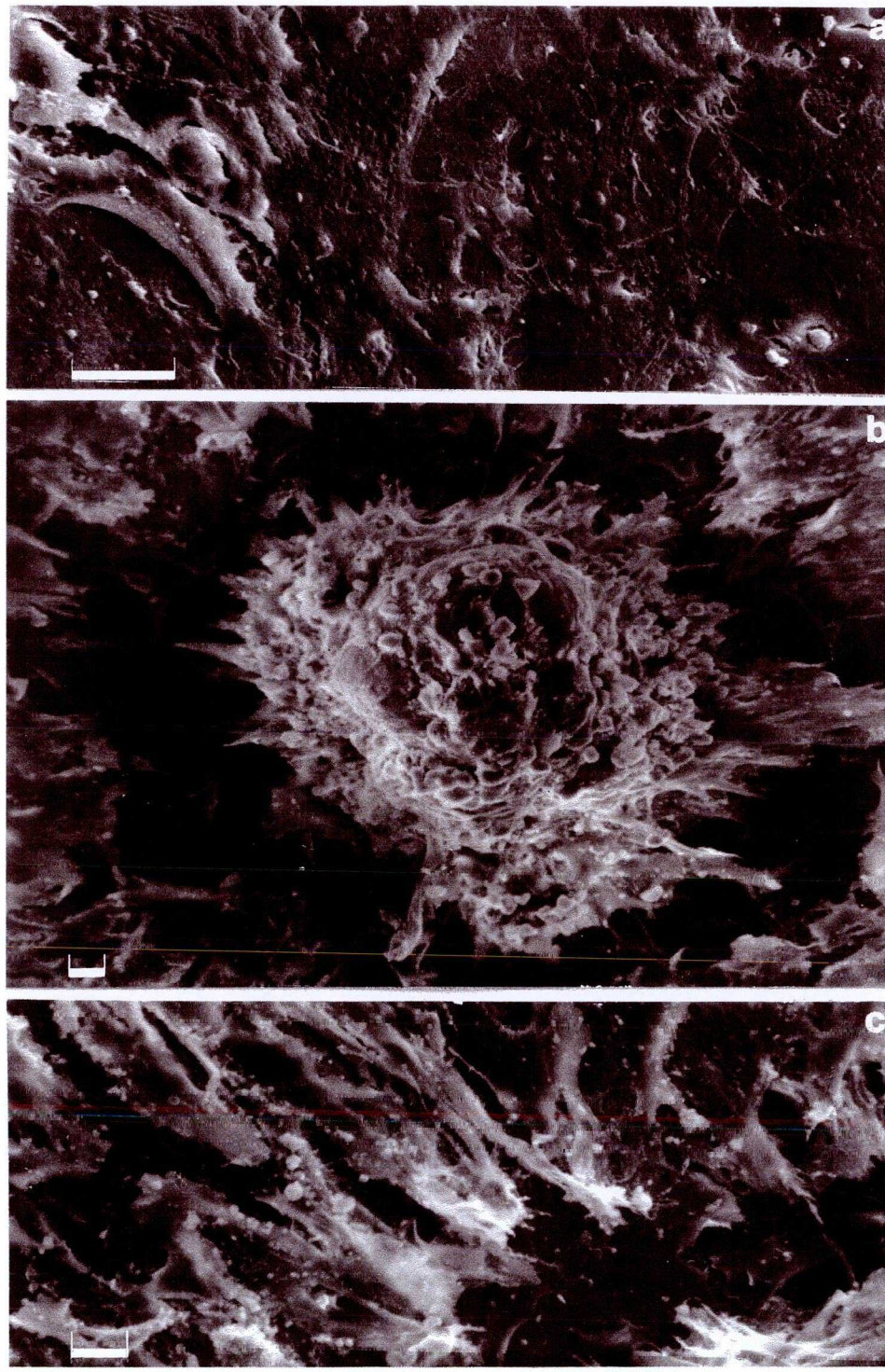


Fig. 6. Scanning electron microscopy of 45T-1 Sertoli cells. **a**: The monolayer of 6-days-old control cultures consisted of flat, polygonal cells. **b**: A spheroid formed under the effect of TNF (1000 U/ml 10 days). **c**: Elongated cells covered by small blebs in the neighborhood of a spheroid (1000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

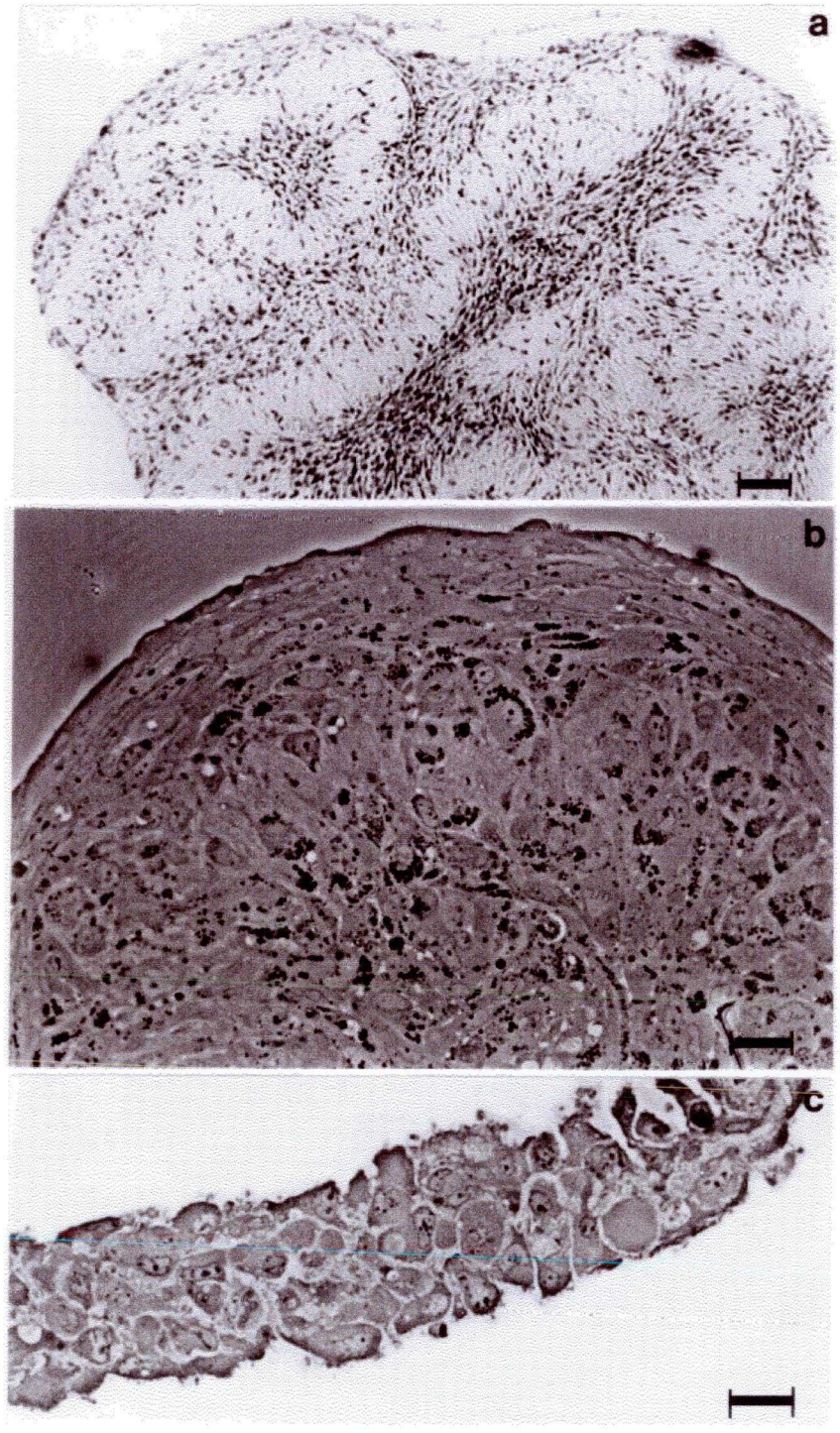


Fig. 7. Sections of frozen (**a**) and aldehyde-fixed, Araldite embedded (**b**) spheroids from cultures exposed to TNF (1000 U/ml) for 21 days. The spheroids are covered by several layers of flat, elongated cells which protrude into the core and divide the inner cell mass into more or less separated areas. The cells contain many lipid droplets. Control cultures (**c**) did not show signs of morphological changes. Bars: 100 μm.

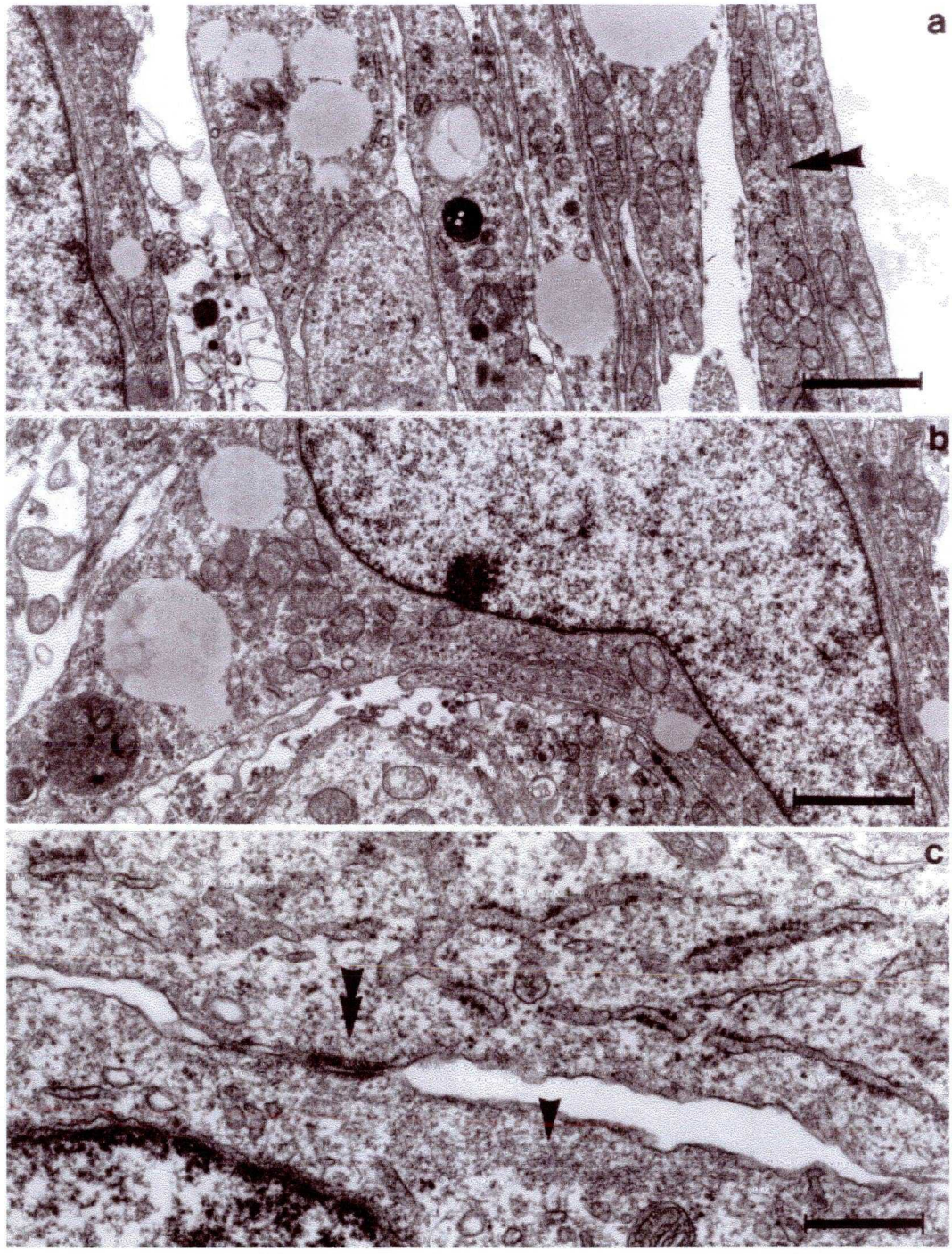


Fig. 8. Electron micrographs from the outer (**a** and **c**) and inner parts (**b**) of a spheroid (TNF 1000 U/ml 14 days). The spheroid is covered by several layers of flat cells. Note the presence of lipid droplets and microfilaments (double arrow in **a**, arrow in **c**); and cell-cell contact (double arrow in **c**). The inner cells are usually large, with cellular debris between the cells. Bars: 20 nm.

2.2. Expression of laminin and desmin are upregulated in TNF treated 45T-1 cells

Sertoli cells participate in the secretion and maintenance of basal membranes surrounding the seminiferous tubules. Production of laminin and collagen, components of 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 [29] and Matrigel was reported to have similar effect on a polyoma T antigen immortalized cell line, very similar to 45T-1 [60]. 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. 9.a and b). Laminin specific immunostaining was negative in control cultures and in non-aggregated cells of TNF treated Sertoli cell monolayers (Fig. 9.c).

The formation of the observed 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 of the control and TNF treated cells. TNF treatment did not cause dramatic changes in the quantity and distribution of these proteins (not shown).

In contrast, desmosomal protein was weakly expressed both in control cultures and in the ones treated with TNF for 24 hours (Fig. 10.a), but 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. 10.b). Six days after TNF treatment when the cells started to assemble into large aggregates and in the fully developed spheroids immunhistological examination proved the high level expression of desmin (Fig. 10.c 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. 8.c).

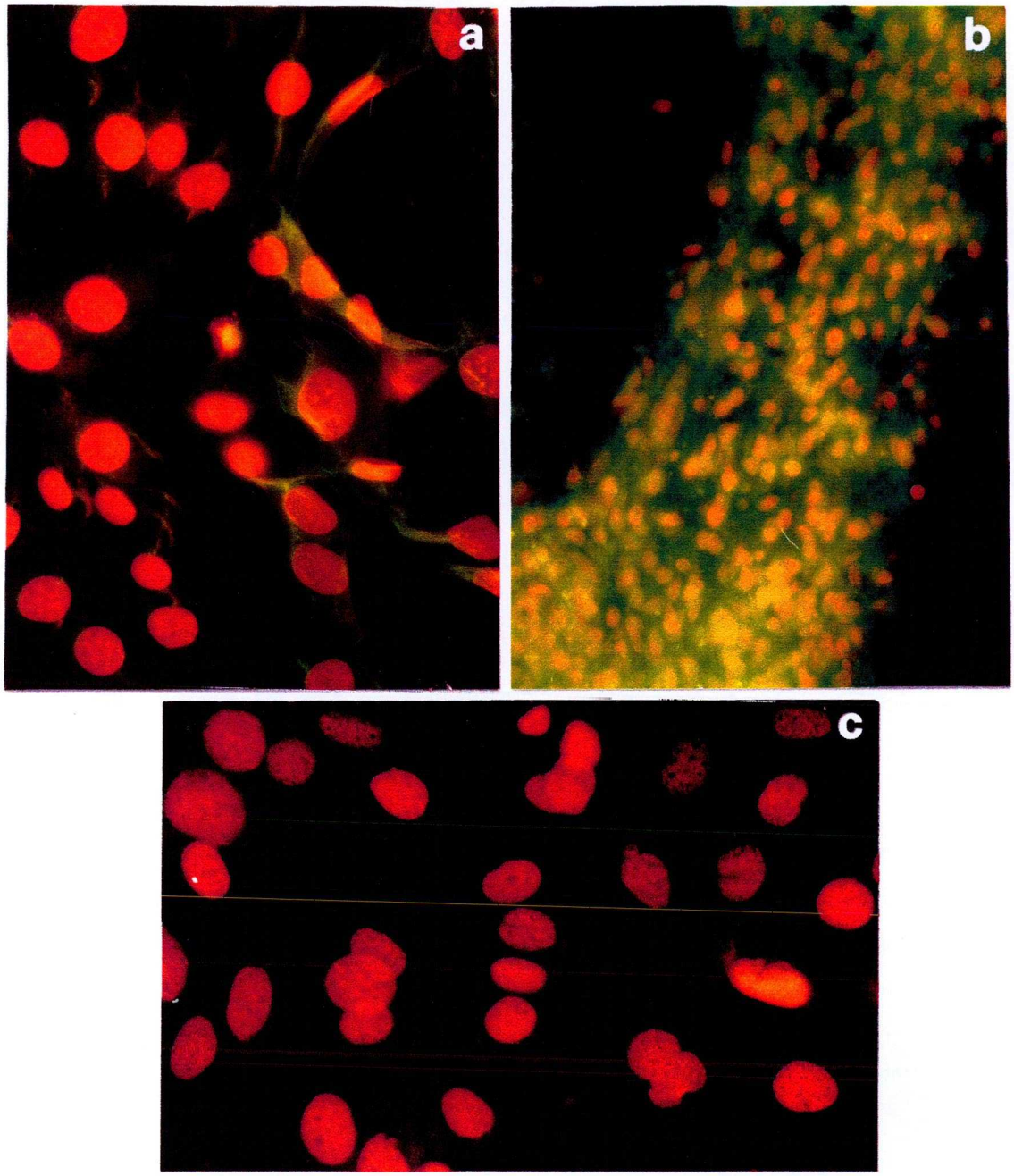


Fig. 9. 45T-1 cells exposed to 1000 U/ml TNF for 3 (**a**) and 12 (**b**) days, stained with antibody for laminin. The nuclei were stained with ethidium bromide. The expression of laminin is weak after short term treatment (**a**), however, intensifies in aggregated cells after 12 days (**b**). Control cells (**c**) show no laminin specific staining.

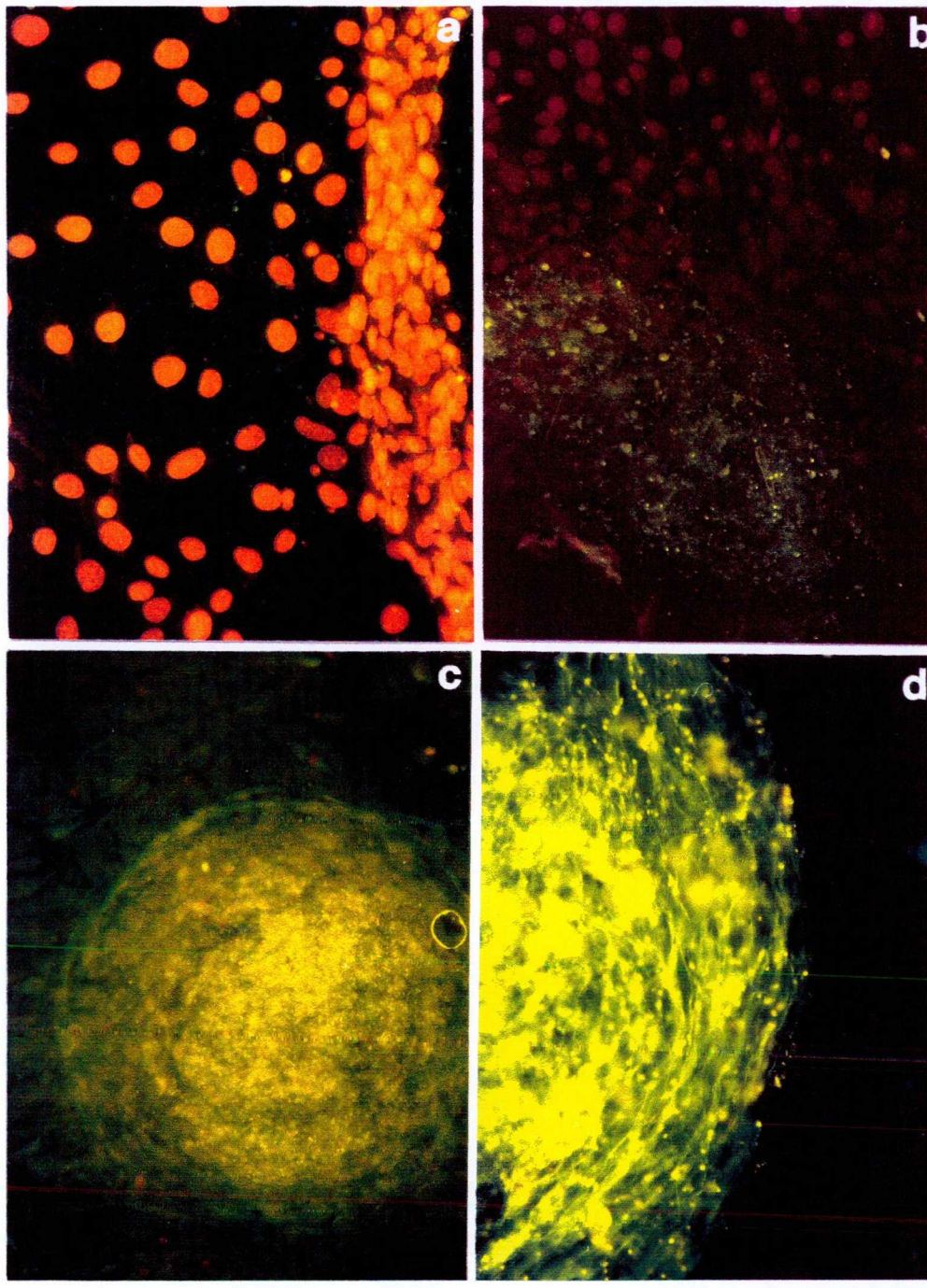


Fig. 10. 45T-1 cultures stained with antibody against desmosomal protein followed by FITC-conjugated secondary antibody as described in Materials and methods. The nuclei were stained by ethidium bromide. **a**: 3-days-old control cells; **b**, **c** and **d**: cultures exposed to 1000 U/ml TNF for 3, 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 (**a**) and in those TNF-treated cells which surround the spheroids (**b** and **c**).

2.3. 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. The 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.

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 or thiol compounds, which block eicosanoid metabolism or 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) induced the aggregation of 45T-1 cells in concentrations of 1 to 10 thousand U/ml, however, no inner structure was observed in these aggregates.

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 and 45-Ti Sertoli cells. The results presented here demonstrate a previously unknown biological activity of TNF that may have substantial physiological importance.

In skeletal muscle cells a general antagonism between proliferation and the aquisition of the differentiated phenotype exists. Consequently, when muscle cells differentiate, they irreversibly withdraw from the cell cycle before they start to synthesize muscle-specific proteins. 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 in 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 haevy chain mRNA expression was reported earlier by Miller et al. [48] in human primary myoblasts. In our experiments in addition to actin and myosin haevy chain, synthesis of myosin light chain and the most important myogenic regulatory factors, MyoD and myogenin were also strongly inhibited by TNF treatment of mouse C2/7 myoblasts. As a consequence, we observed disrupted myofibers in TNF-treated myoblast cells.

Whereas MyoD induce terminal cell cycle arrest during skeletal muscle differentiation, myogenin has a separate function and is required for the formation of differentiated muscle fibers. MyoD expression and activity are both inhibited by active cyclin D1-Cdk 4 complexes [30]. Cell cycle arrest, induced by p21 (and $p27^{KIP1}$) proteins ensures MyoD activity [30], which is normally strengthened by positive autoregulation [72]. 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 cyclin-kinase complexes. Other transcription factors might also have a role: TNF induced formation of (abnormal) myogenin-Jun-D complexes was reported in cachexia [11].

TNF has a pivotal role in the inflammatory response, particulary in its ability to affect the synthesis and degradation of extracellular matrix (ECM) molecules. TNF may also have a physiological role in the coordinated removal and replacement of senescent ECM components during normal tissue homeostasis. It is therefore possible that TNF may also serve similar functions during embryonic development, since this process is characterized by high rates of remodelling of ECM. Interaction of cells with ECM and with surrounding cells plays an important role in cellular positioning, migration, and differentiation during development. The balance between production and degradation of collagen plays a critical role in the development and maintenance of virtually every organ and tissue. Collagen synthesis is almost always inhibited by TNF both *in vitro* [70] and *in vivo* [12]. The presence of collagen fibers in TNF treated myoblast cells suggested another explanation: TNF induced TGF-β production. TNF exerts a negative regulatory effect on collagen gene expression, antagonizing with TGF-β driven upregulation of type I collagen expression. It was reported that myoblast differentiation, in particular, is reversibly inhibited by the action of TGF-β1 and TGF-β2 on primary cultures and established lines of chick, rat, and mouse myoblasts [42, 58]. The mechanism by which TGF-βs inhibit myoblast differentiation is unknown, but it has been noted that an early and persistent response of myoblasts and many other cell types to TGF-β is a marked elevation in the expression of ECM components including fibronectin, collagen, proteoglycans, protease inhibitors, and cell adhesion receptors [2, 31, 34]. However, we could not detect elevated TGF-α message levels in TNF treated cell cultures under any of the experimental conditions tested (not shown).

Our results show, that TNF and its membrane-bound form are able to influence the differentiation of myoblast cells. The physiological significance of the inhibition of myogenesis by TNF remains to be established. One possible function of TNF is prevention of precocious fusion of embryonic myoblast to form postmitotic myotubes during that period in development when extensive myoblast proliferation is essential to form the initial muscle mass. Under these circumstances, there is a relatively high density of myogenic cells, but they do not fuse. It is possible that TNF or related agents might prevent differentiation.

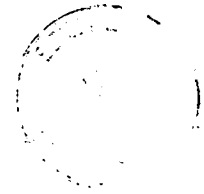
Another possible action of TNF might be in regulating the formation of satellite cells in skeletal muscle. These cells, which are localized under the primary muscle membrane, are separated from the main body of the muscle by a second plasma membrane. Satellite cells contain the only nuclei in mature muscle capable of further DNA synthesis, and these cells are activated to form additional myogenic cells during muscle regeneration, as well as in normal growth of skeletal muscle. Satellite cells have the potential to proliferate under condition of muscle injury. Therefore, on a functional basis, satellite cells are developmentally indistinguishable from embryonic myoblasts in that both serve as myogenic precursors. The target of TNF action may be the satellite cells in muscle tissue. There are two populations of precursor cells: committed satellite cells, which are ready for immediate differentiation without preceding cell division, and stem satellite cells, which undergo mitosis before providing one daughter cell for differentiation and another for future proliferation [62]. Only 50% of the satellite cell descendants enter the phase of terminal differentiation [82]. Thus, under conditions of muscle injury, TNF may function to inhibit satellite cell differentiation while sparing differentiated muscle tissue.

Accumulation of mitotically active myoblast is necessary for the muscle regeneration that could also be important in wound healing. In this situation, the differentiation-inhibiting activities of TNF would prevent premature formation of postmitotic myotubes until adequate muscle mass had accumulated. Once a myoblast withdraws from the cell cycle, its position in relationship to the adjacent cell is essentially determined. It requires a sufficient population of myogenic cells in the neighborhood to allow fusion and the production of the final muscle. Hence it is important not to initiate this process until the cell is in the proper position and in the company of a sufficient population of other myogenic cells. In short, it needs some feedback from the environment. It is possible, that local elevations of TNF might prevent the fusion of putative satellite cell nuclei into the main body of the muscle during initial myogenesis, thus making these nuclei available for activation at a later time.

In addition to wound healing and embryonic development, TNF plays a critical role in a variety of pathophysiological processes. 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, common findings in patients with AIDS, cancer and diseases characterized by chronic inflammation [11]. 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. The heart is a TNF-producing organ where both myocardial macrophages and cardiac myocytes themeselves synthesize TNF [45]. Accumulating evidence indicates that myocardial TNF is an autocrine contributor to myocardial dysfunction and cardiomyocyte death in ischemia-reperfusion injury, sepsis, chronic heart failure, viral myocarditis, and cardiac allograft rejection. Locally produced TNF contributes to postischemic myocardial dysfunction via direct depression of contractility and induction of myocyte apotosis [45]. Abnormalities in the metabolism of muscle connective tissue may lead to pathological alterations and loss of muscle functions, e.g., in muscular dystophy which is associated with a fibrotic proliferation of connective tissue [61]. At present there is no satisfactory treatment for these complications, which contribute significantly to the morbidity and mortality of these patients. Thus modulation of TNF in these diseases represents a realistic goal for clinical medicine.

We have shown also 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. When Sertoli cells grow at the border of the aggregates, they acquire a columnar appearance with polarization of their cytoplasma. The nucleus is then located in the basal part of the cytoplasm, close to the adjacent cells. This aggregation was accompanied by TNF-dependent formation of highly organized inner structures and upregulation of desmosomal protein and laminin detected by immunohistochemistry. We observed cell-to-cell interconnections and adhering junctions like desmosomes, that are more characteristic of tissues than of cell cultures. *In vivo*, the appearance of Sertoli-Sertoli junctional complexes occurs in most mammals at about the same time as the start of meiosis by germ cells. This barrier formation may have a role in the onset of spermatogenesis. So far, the factors responsible for stimulating formation of junctional complexes between adjacent Sertoli cells have not been conclusively identified. Control cultures were arrested at confluency and monolayers could be maintained for several weeks



without overgrowth. While aggregation was also induced by the functionally related cytokine, IL-1β, organization of the inner structures was triggered only by TNF.

Laminin and desmin 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* [29, 60], it is quite possible, that accumulation of TNF-induced laminin provoked further steps of aggregation and morphological changes.

Hofmann et al. [33] observed that when cells representing each of the immortalized main cell types of the testis (peritubular, Leydig, Sertoli, and germ cells) are cocultured, they are able to reaggregate in a characteristic and reproducible manner, forming structures resembling two-dimensional seminiferous tubules *in vitro*. Similarly, co-cultures of myoid and Sertoli cells form large cell clusters in response to TGF-β1 [69]. It is also prooved that testis-derived cells transplanted into the testis of an infertile mouse will colonize seminiferous tubules and initiate spermatogenesis, if the donor population containes both germ cells and Sertoli cells [9].

Since in our experiments Sertoli cells form tissue-like structures without the participation of germinal or any other cell types of the testis, it seems likely that (one of) the factor(s) responsible for the organizing properties of testicular cells is the TNF. TNF was detected in spermatogenic cells and mRNA of the 55 kDa human TNF alpha receptor is present in Sertoli cells [20]. The 26 kDa transmembrane form of TNF is present on the surface of germinal cells [51]. 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 [5]. 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.

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 [22]. The finding that the bFGFR-1 is developmentally regulated in the testis under the control of FSH, TNF and FGF [40] also supports the role of TNF in testicular development.

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

regulation of seminiferous tubules. The observations presented here indicate that the permanent cell lines have retained their organogenic potential.

It is not totally unexpected that TNF has opposing effects on differentiation and morphogenic processes of different cell types. Both positive and negative effects of TNF are known from the literature. TNF inhibits epithelial differentiation and morphogenesis in the mouse metanephric kidney *in vitro.* Addition of recombinant TNF to metanephric organ culture induced a decrease in growth and development, with inhibition of ureteric bud branching and nephron formation [15]. TNF caused differentiated fat cells to lose stainable lipid and to revert to an indifferent phenotype [73] and it is a significant (negative) effector of proliferation and differentiation in inner cell mass-derived embryonic stem cells [81]. TNF drastically reduced the capacity of granulosa cells to differentiate upon FSH stimulation and to respond to LH during the physiological ovarian follicular maturation [17].

In contrast, TNF could induce the *in vitro* differentiation and neurite formation of the neuroblastoma cell line N103 in a dose-dependent manner [53]. TNF stimulated pulmonary branching, morphodifferentiation and histodifferentiation [35], and induced clustering in ovarian theca-interstitial cells (TIC) *in vitro* [85]. TIC were organized into spheroid-shaped clusters consisting of concentric layers of cells. TNF acting alone, or in concert with other factors, may function as a thecal (and/or granulosa cell) organizing factor, that attracts these cells to the oocyte during follicular development [85]. (Thecal cords express TNF and Sertoli cells are the testicular homolog of ovarian granulosa cells.)

Finally, a role for TNF in development is also supported by the findings about the teratogenic effects of thalidomide. Thalidomide was administered to women in the first trimester of pregnancy, as a treatment for nausea, in the late 1950s and 1960s. However, as is well known, it often resulted in severe defects in the fetuses. Recently it has come to light that thalidomide has the ability specifically to induce degradation of TNF mRNA [52]. Thus, it is possible that thalidomide could excepted its teratogenic effect by inhibiting developmentally regulated TNF expression.

Summary

A central question in developmental biology pertains to how interacting groups of cells and molecules give rise to tissues exhibiting specialized forms and functions. It is hoped that these results may provide the basis for further descriptive and functional studies that will help to elucidate the role of TNF-like proteins in embryonic development. Whether the *in vitro* data presented here reflect a potential physiological (or pathophysiological) role of TNF in myogenesis and in the male gonad function requires further studies. Nontheless, these observations illustrate that a cytokine can have many effects and that the actions of a single peptide on a single cell type can be very complex.

We have shown that:

- both soluble and transmembrane form of TNF facilitates C2/7 mouse myoblast cell migration and aggregation *in vitro*,
- TNF enhances the proliferating activity of the C2/7 myoblast cells,
- TNF inhibits the expression of myofilament components, like α-skeletal actin, myosin heavy chain and myosin light chain in C2/7 myoblast cells,
- TNF inhibits the expression of the genes of myogenic regulatory factors: MyoD and myogenin in C2/7 myoblast cells,
- as a consequence, TNF inhibits myofilament formation and differentiation of C2/7 myoblast cells,
- TNF triggers migration and aggregation of 45-T1 Sertoli cells *in vitro*,
- TNF induces highly organised 3-dimensional structures of 45-T1 Sertoli cells that contain several adhering junctions and desmosomes resembling to seminiferous tubules of testis,
- expression of laminin and desmin are highly upregulated in TNF-treated 45-T1 Sertoli cells,
- TNF has no effect on the presence of other cytosceletal elements, like alpha- and beta-tubulin, cytoskeletal actin, cytokeratin, vinculin and vimentin in any conditions we tested.

Acknowledgements

I would like to thank to Prof. Ernő Duda for his scientific guidance and the opportunity to work in his group.
I thank Erzsébet Kúsz and Andrásné Borka for the excellent technical assistance.
I would like to thank also to Zsolt Rázga, János Kovács, Tivadar Mikó and Miklós Sass for their work on the electronmicroscopic and immunohistochemic part of this project.

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