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Morphological, Biochemical, and Molecular Biological Characterization of a Rat Rhabdomyosarcoma Cell Line during Differentiation Induction *In Vitro*

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BA-HAN-1C is a clonal rat rhabdomyosarcoma cell line consisting of proliferating mononuclear tumor cells, some of which spontaneously fuse to form terminally differentiated postmitotic myotubelike giant cells. Exposure to retinoic acid resulted in an inhibition of proliferation and a marked increase in cellular differentiation. The number of myotubelike giant cells significantly increased, and about 30% of the mononuclear tumor cells exhibited morphological features of rhabdomyogenic differentiation which were not observed in the mononuclear cells of untreated cultures. Morphological differentiation was paralleled by an increase in total creatine kinase activity as a biochemical marker of differentiation. These effects of retinoic acid were preceded by an increased expression of proto-oncogene *raf* and transient expression of proto-oncogene *fos*. The maximum level of *fos* expression was observed at 15 min and of *raf* at 12 hr after exposure to retinoic acid. No expression of the proto-oncogenes *src, myb, myc, ros, mos, erbA,* and *erbB* was detected.

Introduction

Retinoic acid has been shown to be a very potent inducer of differentiation, suppressing proliferation and enhancing differentiation in various tumor cell lines (*1*). Attention has, however, been focused mainly on carcinomas, melanomas, and leukemias, whereas sarcomas have received less attention, partly because cell-type specific markers of differentiation are not readily available in many established sarcoma cell lines. We report here on the effects of retinoic acid on the clonal rat rhabdomyosarcoma cell line BA-HAN-1C. This cell line closely imitates embryonic rhabdomyogenesis (*2*). It consists of myogenically committed but morphologically undifferentiated mononuclear stem cells. Some of these mononuclear tumor cells spontaneously fuse to form multinuclear myotubelike giant cells with ultrastructural features of rhabdomyogenic differentiation. These myotubelike giant cells were shown to have irreversibly withdrawn from the mitotic cycle and to represent terminally differentiated postmitotic tumor cells (*3*).

Materials and Methods

Cells and Culture. The clonal cell line BA-HAN-1C was derived from a dimethylbenzanthracene-induced rhabdomyosarcoma in the rat (*2*). The standard growth medium was Dulbecco-Vogt's modified Eagle medium (DMEM, Gibco Europe, FRG), supplemented with 10% heat-inactivated fetal calf serum, penicillin, and streptomycin. Cell counts were performed with the Neubauer hemocytometer chamber. For differentiation induction, the stock solution of 5 mM retinoic acid (Serva, FRG) in 95% ethanol was diluted in standard growth medium.

In Vitro *Morphology.* For transmission electron microscopy, the tumor cells were seeded on glass cover slips. After incubation for 5 days, the tumor cells were fixed *in situ* and further processed as previously described (*2*).

Fusion Assay. On the bottom of culture flasks four arbitrarily located fields were marked. The area marked out by these four fields was 1/32 the growth area of the culture flask. The number of myotubelike giant cells in the marked fields was counted by phase contrast microscopy at intervals of 24 hr. At the end of the observation period of 72 hr, the total number of tumor cells was determined in each culture flask.

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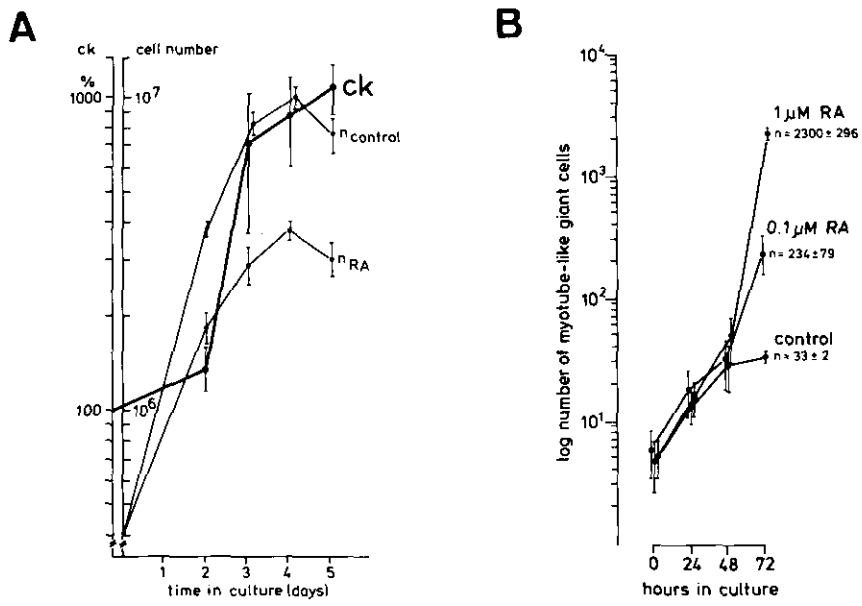

FIGURE 1. (*A*) Growth behavior and creatine kinase activity of BA-HAN-1C after exposure to 1 μM retinoic acid. The creatine kinase activity is significantly different from the control 72 hr after exposure to retinoic acid ($p < 0.05$; Wilcoxon test for unpaired samples). The difference between the growth curves is statistically significant ($p < 0.001$; analysis of variance with two independent factors). ck, Creatine kinase activity; $n_{control}$, cell number in standard growth medium; n_{RA}, cell number in growth medium supplemented with retinoic acid. (*B*) Absolute number of myotubelike giant cells of BA-HAN-1C. Each value represents the mean ± SD of five experiments.

Total Creatine Kinase Activity. Tumor cells were disrupted by sonication, and the total creatine kinase activity, which was used as a biochemical marker of differentiation (*4*), was determined on an Olympus AU 5031 analyzer using the CK-test (NAC-activated) from Merck (Darmstadt, FRG).

Northern Blot Analysis. Total cellular RNA was isolated by the guanidine isothiocyanate method (*5*) and analyzed as previously described (*6*). Insert DNA for probing c-*onc* genes was obtained from the following plasmids: p-c-*fos* (Exon IV), insert: PvuII/SacI; p-c-*myb*, insert: Eco RI; p-EH-hu-c-*myc*, insert: SstI; p-*erbB*/*erbA*; p-*src*, insert: PvuII; p-*ros*, insert: EcoRI/PvuII; p-Ki-*ras*, insert: EcoRI; p-*raf*, insert: XhoI/SstII; p-*mos*, insert: XbaI/HindIII. All plasmids have been described previously (*7*). Insert DNA of pHKI encoding the human retinoic acid receptor (hRARα) was used as a probe for retinoic acid receptor (*8*).

Southern Blot Analysis. Genomic DNA was isolated from BA-HAN-1C cells, skeletal muscle, and liver. Chromosomal DNA was digested with EcoRI to completion, and 10 μg digested DNA in each lane was separated electrophoretically on 0.7% agarose gel, denatured in 0.5M NaOH/1 M NaCl, neutralized, transferred to nitrocellulose, and hybridized to the *raf*-specific probe. Hybridization conditions were the same as previously described (*6*).

Cycloheximide Inhibition Studies. Cycloheximide (5 μg/mL final concentration) was added to growth medium which was exchanged after 4 hr in order to avoid cytotoxic effects. Two kinds of studies were performed. Cycloheximide was either given at the same time as retinoic acid or 4 hr before retinoic acid.

Results

Morphological and Biochemical Aspects of Differentiation Induction

Exposure to 1 μM retinoic acid resulted in a statistically significant ($p < 0.001$) inhibition of proliferation (Fig. 1*A*). The tumor cells were aligned in a more orderly arrangement 72 hr after exposure to 1 μM retinoic acid (Fig. 2*B*) and piling up was significantly reduced as compared to the criss-cross growth pattern under standard growth conditions (Fig. 2*A*). Transmission electron microscopy revealed that about 30% of the mononuclear tumor cells (Fig. 2*D*) exhibited irregular bundles of thick myofilaments (12–15 nm in diameter) and thin myofilaments (6–8 nm in diameter), which had never been observed in the mononuclear tumor cells under standard growth conditions (Fig. 2*C*). Furthermore, exposure to retinoic acid resulted in a dose- and time-dependent increase in the absolute number of myotubelike giant cells (Fig. 1*B*). The ratio between the number of myotubelike giant cells (in 1/32 the growth area of the culture flask) and the total number of tumor cells per culture flask was calculated. For this ratio, a statistically significant ($p < 0.001$), dose-dependent increase became evident from $4 \times 10^{-6} \pm 0.0$ (control) to $30 \times 10^{-6} \pm 13 \times 10^{-6}$ (0.1 μM retinoic acid) and $310 \times 10^{-6} \pm 33 \times 10^{-6}$ (1 μM retinoic acid) after 72 hr. Morphological differentiation induction was paralleled by a statistically significant increase ($p < 0.05$) in total creatine kinase activity (Fig. 1*A*) when compared to the control.

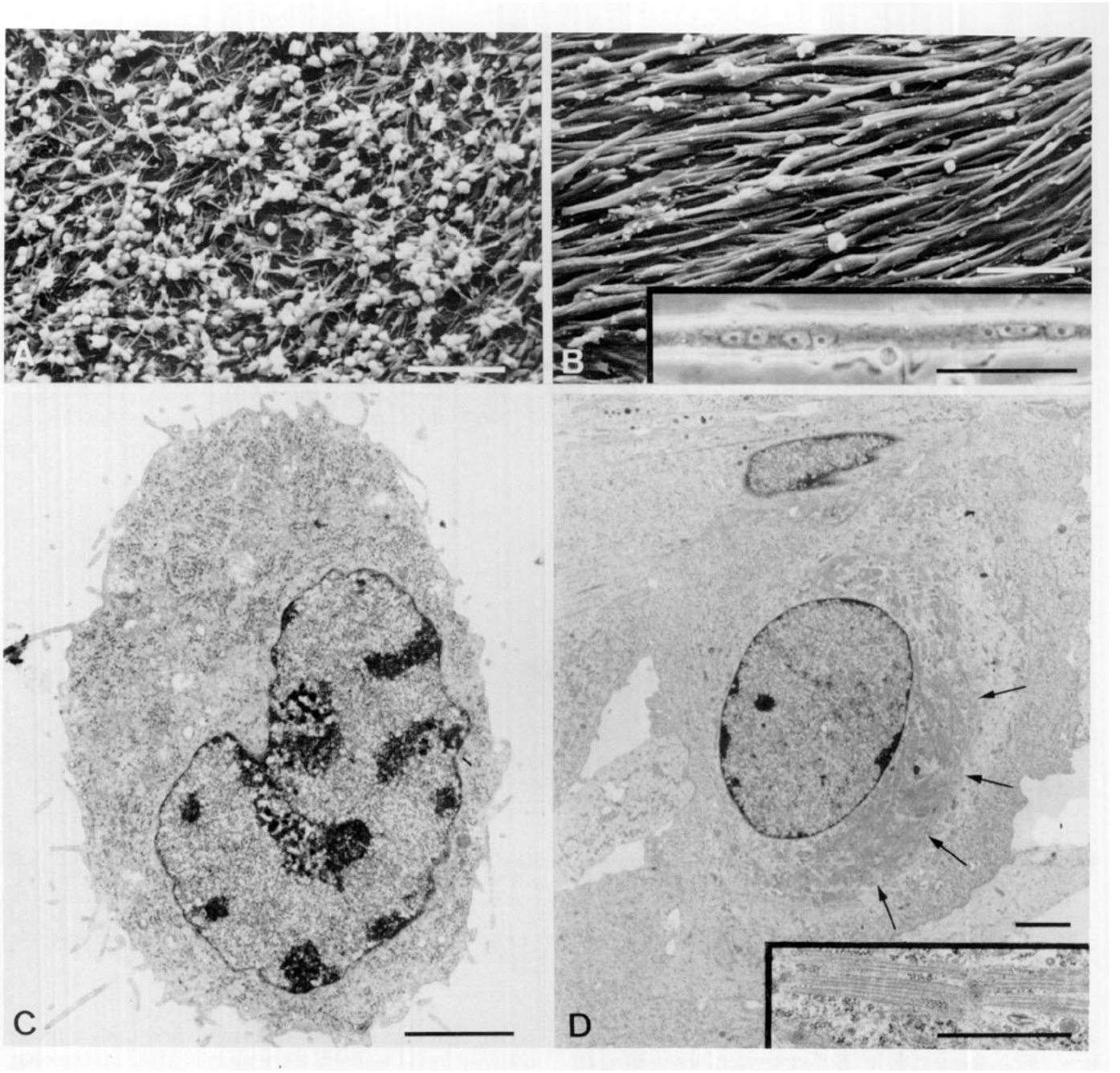

FIGURE 2. Morphology of BA-HAN-1C tumor cells. (*A*) Criss-cross growth pattern of mononuclear tumor cells in standard growth medium. Bar = 100 μm. (*B*) More regular arrangement of spindle-shaped tumor cells often exhibiting multiple nuclei (inset) after exposure to retinoic acid. Bar = 100 μm. (*C*) Mononuclear tumor cell in standard growth medium without features of rhabdomyogenic differentiation. Bar = 2 μm. (*D*) Mononuclear tumor cell after exposure to retinoic acid with irregular bundles of thick and thin myofilaments (arrows, inset). Bar = 2 μm; inset, bar = 0.5 μm.

Molecular Biological Aspects of Differentiation Induction

The initial screening for proto-oncogene expression in BA-HAN-1C (Fig. 3*A*) revealed that the proto-oncogene *raf* was expressed at a low steady-state level under standard growth conditions. The expression of *raf* markedly increased after exposure to retinoic acid for 6 days (Fig. 3*A*). Furthermore, a low expression level of the proto-oncogene *ras* was detected 6 days after exposure to retinoic acid (Fig. 3*A*). No expression was detected for the proto-oncogenes *fos*, *src*, *myb*, and *ros* (Fig. 3*A*), as well as *myc*, *mos*, *erbA*, *erbB* (data not shown) before or 6 days after exposure to retinoic acid.

Kinetic studies on the expression of the proto-oncogene *raf* (Fig. 3*D*) showed a maximum expression level 12 hr

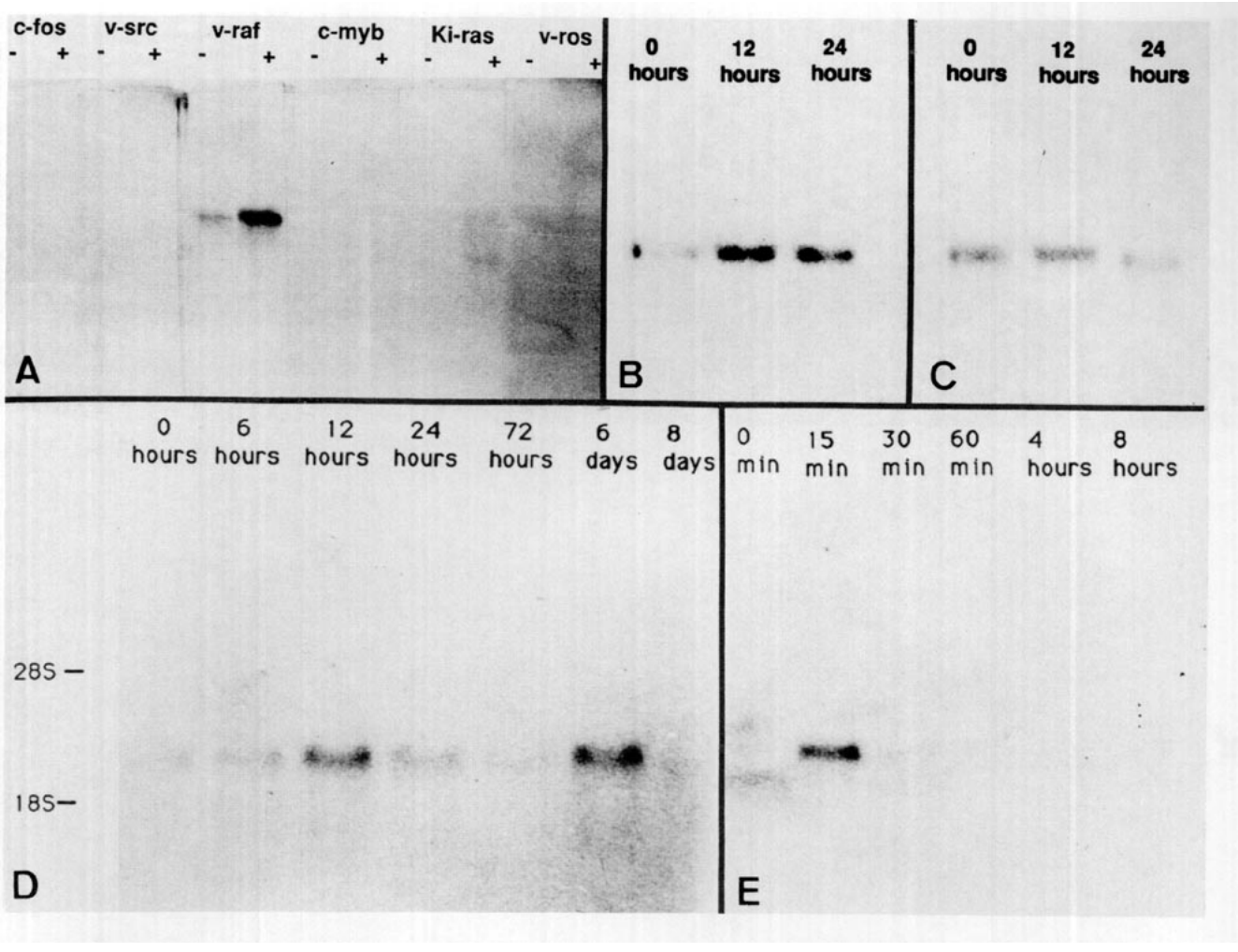

FIGURE 3. (*A*) Northern blot analysis of proto-oncogene expression in BA-HAN-1C tumor cells before (−) and after (+) exposure to 1 μM retinoic acid for 6 days. Medium change with fresh retinoic acid was performed on day 5. (*B,C*) Proto-oncogene *raf* expression of BA-HAN-1C tumor cells by Northern blot analysis after simultaneous exposure to retinoic acid and cycloheximide (*B*) or after preincubation with cycloheximide for 4 hr followed by exposure to retinoic acid (*C*). (*D,E*) Kinetic studies on the expression of the proto-oncogenes *raf* (*D*) and *fos* (*E*) by Northern blot analysis at indicated time intervals after exposure of BA-HAN-1C tumor cells to 1 μM retinoic acid; note second increase in *raf* expression on day 6 after medium change with fresh retinoic acid. Each lane contained 15 μg of total RNA.

after exposure to retinoic acid, which declined below basal level within the next 60 hr. *Raf* expression increased again following medium change with fresh retinoic acid. Kinetic studies on the expression of the proto-oncogene *fos* showed a fast response to retinoic acid within 15 min (Fig. 3*E*). The expression of *fos*, however, rapidly declined to undetectable levels within the next 45 min. Control experiments showed that medium change without retinoic acid was not sufficient to induce the proto-oncogenes *fos* and *raf* (data not shown).

When cycloheximide, a potent inhibitor of protein synthesis, was given at the same time as retinoic acid, the increase in the expression of the proto-oncogene *raf* was not impaired (Fig. 3*B*). However, when cycloheximide was added to the cells 4 hr before the addition of retinoic acid, no increase in proto-oncogene *raf* expression was observed (Fig. 3*C*). The presence of retinoic acid receptor mRNA could be demonstrated in BA-HAN-1C tumor cells before and after exposure to retinoic acid using a probe obtained from the insert of the plasmid pHKI (*8*) encoding the human retinoic acid receptor hRARα (data not shown). Interestingly, retinoic acid receptor mRNA could not be detected in normal rat skeletal muscle. Studies on the genomic arrangement of the proto-oncogene *raf* by Southern blotting revealed no difference between hybridizing DNA fragments in the chromosomal DNA of BA-HAN-1C tumor cells, normal rat skeletal muscle, and rat liver (data not shown).

Discussion

Numerous hypotheses on the molecular mode of action of retinoic acid in the control of proliferation and differentiation have been proposed (*1*). Recently, it has been suggested that retinoic acid affects the expression of proto-oncogenes involved in the control of proliferation

and differentiation (*9*). This observation is confirmed by our investigations that demonstrate that the effects of retinoic acid on the proliferation and differentiation of BA-HAN-1C tumor cells were preceded by remarkable changes in the expression of the proto-oncogenes *fos* and *raf*. The altered expression of the proto-oncogene *raf* is of particular interest. The physiological function of *raf* is still obscure even though the biochemical function of its gene product, a serine/threonine-specific kinase, has been described (*10*).

The induction of c-*raf* in BA-HAN-1 cells upon exposure to retinoic acid during an early phase of rhabdomyogenic differentiation is reported for the first time. The direct linkage between retinoic acid and *raf* expression was confirmed by the cycloheximide experiments. When cycloheximide, a potent inhibitor of protein synthesis, was given at the same time as retinoic acid, no change in the increased expression of the proto-oncogene *raf* was observed. This result indicated that the *de novo* synthesis of proteins, especially of the fos protein, was not necessary to increase the expression of the proto-oncogene *raf*. In contrast, when cycloheximide was given 4 hr before retinoic acid, *raf* expression did not increase. This observation suggested the depletion of a cellular protein, e.g., the retinoic acid receptor protein, which is supposedly indispensable for the signal transduction of retinoic acid. The existence of such a receptor protein in the cell line BA-HAN-1C was also suggested by the presence of the mRNA homologous to human retinoic receptor hRARα.

Induction of the proto-oncogenes *fos* and *raf* by retinoic acid preceded biochemical and morphological differentiation in the rhabdomyosarcoma cell line BA-HAN-1C. This cell line may therefore serve as an appropriate experimental system to further elucidate the regulatory events taking place during differentiation induction with retinoic acid.

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