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

by Claus D. Gerharz,* Johannes Doehmer,† Horst Mayer,‡ Franz Oesch,† and Helmut Gabbert*  

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 ras and transient expression of proto-oncogene fos. The maximum level of fos expression was observed at 15 min and of ras at 12 hr after exposure to retinoic acid. No expression of the proto-oncogenes src, myb, myc, ras, 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 These myotubelike giant cells were shown to have irreversibly withdrawn from the mitotic cycle and to represent terminally differentiated postmitotic tumor cells (3).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 1/4 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.
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 5081 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: EcoR I; p-EH-hu-c-myC, insert: StI; p-erB/erB A; p-src, insert: PvuII; p-ros, insert: EcoRI/PvuII; p-Ki-ros, insert: EcoRI; p-rf, 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 rf-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. 1A). The tumor cells were aligned in a more orderly arrangement 72 hr after exposure to 1 μM retinoic acid (Fig. 2B) and piling up was significantly reduced as compared to the criss-cross growth pattern under standard growth conditions (Fig. 2A). Transmission electron microscopy revealed that about 30% of the mononuclear tumor cells (Fig. 2D) 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. 2C). Furthermore, exposure to retinoic acid resulted in a dose- and time-dependent increase in the absolute number of myotubelike giant cells (Fig. 1B). The ratio between the number of myotubelike giant cells (in 1/50 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 × 10⁻⁶ ± 0.0 (control) to 30 × 10⁻⁶ ± 13 × 10⁻⁶ (0.1 μM retinoic acid) and 310 × 10⁻⁶ ± 33 × 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. 1A) when compared to the control.
DIFFERENTIATION INDUCTION IN A RHABDOMYOSARCOMA CELL LINE

Molecular Biological Aspects of Differentiation Induction

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

Kinetic studies on the expression of the proto-oncogene \textit{raf} (Fig. 3D) 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 \( \mu M \) 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 \( \mu M \) retinoic acid; note second increase in \( raf \) expression on day 6 after medium change with fresh retinoic acid. Each lane contained 15 \( \mu g \) of total RNA.

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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