Differentiation and Dedifferentiation of the Human Monocytic Leukemia Cell Line, U937

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ABSTRACT. U937 cells were differentiated into macrophages after being treated with 12-o-tetradecanoyl-phorbol-13-acetate (TPA) for the first two days and dedifferentiated with daily medium renewal for 10 days. Cell proliferation slowed down and the number of cells reached the maximum level on day 2. By day 4, all of the cells had spread and attached firmly to the culture dish, and more than 90% of the cells expressed the Fc-receptor and produced superoxide anion. From there on, the number of adherent, living cells decreased gradually to about half the initial count. Most of the cells eliminated from the culture by cell death were in the S phase at the time of TPA treatment. After day 8, the number of cells expressing macrophage-specific phenotypes gradually decreased, cell adhesion was weakened, and at the same time, DNA synthesis was initiated anew. The cells became round and began to proliferate as floating cells on days 9 to 10, and thereafter they became sensitive to the second round of TPA treatment. On the basis of all the results taken together, it is suggested that fully differentiated U937 cells were dedifferentiated after being cultured with frequent medium renewal.

MATERIALS AND METHODS

Cell culture. U937, a human monocytic leukemia cell line, was obtained from Dr. K. Nose of the Institute of Medical Science, University of Tokyo, and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; GIBCO, New York, USA) at 37°C in a humidified atmosphere of 95% air, 5% CO₂.

Cells were harvested and suspended in 0.1 M sodium citrate containing 0.1% crystal violet, and then the stained nuclei were counted.

For differentiation-dedifferentiation experiments, exponentially proliferating U937 cells were collected through centrifugation, suspended in a medium containing 0.1 μg/ml TPA at a concentration of 4 × 10⁶ cells/ml, seeded in 35-mm culture dishes and incubated at 37°C for 48 h. After treatment with TPA, differentiated, adherent cells were washed twice with calcium magnesium-free phosphate-buffered saline (PBS⁻), fed normal medium and cultured for 10 more days with daily renewal of the medium. Material floating in the culture supernatant was also discarded on the first 9 days of the experiment since in preliminary experiments it had proven to be cell...
debris or dead cells. Dedifferentiated floating cells on and after day 10 of the experiment were collected through centrifugation and returned to the original dishes after the renewal of the medium.

**Measurement of DNA synthesis through $^3$H-TdR incorporation.** Sample cells were incubated in culture medium containing 10 mM HEPES (pH 7.4) for 30 min to stabilize the culture condition; then 0.1 //Ci/ml $^3$H-TdR was added, and the incubation continued for 1 h. To stop the incorporation, 0.01% NaN$_3$ was added and the culture dishes were transferred onto ice. Alkaline-resistant radioactivity in 5% trichloroacetic acid-insoluble materials collected on glass filters was measured with a liquid scintillation spectrometer.

**Assay of nitroblue tetrazolium (NBT) reducing activity.** Sample cells were incubated in culture medium containing 0.55 mg/ml NBT and 0.1 µg/ml TPA at 37°C for 30 min as described elsewhere (2).

**Assay of Fc receptor (FcR)-mediated phagocytosis.** Sheep

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**Fig. 1.** Morphological changes during differentiation and dedifferentiation. Cells were cultured as described in MATERIALS AND METHODS. Cells of the same site were monitored with phase-contrast microscopy and photographed on the 2nd (A), 5th (B) and 8th (C) day of incubation. The thick line on the left side of each photograph is a scratch mark on the dish ascertaining the field of view. Letters a, b, and c with arrows in B and C indicate corresponding cells identified by taking photos at 24h intervals. ×210.

**Fig. 2.** Course of differentiation and dedifferentiation. U937 cells were seeded at a concentration of $4 \times 10^5$/ml, treated with TPA (0.1 µg/ml) for 2 days, and cultured for 10 more days with daily renewal of the medium. (A) Changes in cell number. Nuclei were stained with 0.1% crystal violet in 0.1 M citrate and counted. (B) Assay of $^3$H-TdR incorporation. Cells were labeled with $^3$H-TdR (0.1 µCi/ml) for 30 min in a medium containing TPA (days 0 and 1) and one not containing TPA (days 2 to 12). An asterisk on the ordinate in (B) indicates the value for the culture when it was growing exponentially.
red blood cells (SRBC) were coated with rabbit anti-SRBC antibody and a quantity of $1 \times 10^8$ SRBC was added to U937 cells and incubated at 37°C for 45 min. Floating cells were collected in glass tubes. To lyse the free SRBC and those located on the cell surface, 2.5 volumes of distilled water were added and mixed for 15 sec, followed by the immediate addition of 0.25 volume of $10 \times PBS^-$; The addition of 1.25% glutaraldehyde fixed cells on the surface of the dish, and the cells with ingested SRBC could be counted.

Cytofluorometry. Cytofluorometry was carried out as described previously (9), using an EPICS-V cell sorter (Coulter Electronics Inc.).

Staining with anti-BrdU antibody. TPA-treated cells were cultured according to the standard procedure of this study except that the cells were seeded in dishes containing cover slips. After a medium change, bromodeoxyuridin (BrdU, 100 μM) was added to the culture, which was then incubated for 12 hr. Cells on cover slips were washed in PBS $^-$ and fixed in 70% ethanol for 15 min. After washing 3 times in PBS $^-$, cells were incubated in 4N HCl for 10 min at room temperature and then washed 4 times in PBS $^-$; FITC-conjugated anti-BrdU antibody (Becton Dikinson, dilution 1:3) was applied to the cells, which were then incubated for 30 min at room temperature. After having been washed 3 times in PBS $^-$, the cells were mounted on glass slides with Gel Tol (Lipshaw).

BrdU-labeled, growing cells were spread on glass slides with centrifugal cell collector (Tommy Co. Ltd.) and stained with anti-BrdU antibody as described above.

Autoradiography. Cells were pulse-labeled with $^3$H-TdR, at a concentration of 0.1 μCi/ml, 1 μCi/ml, or 10 μCi/ml for 1 h, washed PBS $^-$, treated with TPA and cultured according to the standard procedure of this study, except that cells were seeded in dishes containing coverslips. At the given time, cells on the coverslips were fixed and washed 3 times with a 3:1 combination of ethanol and acetic acid, dipped in a 1:1 dilution of nuclear track emulsion (NR-M2, Sakura), exposed for 4 days at 4°C and developed (Konidol, Sakura) for 8 min at 20°C. The number of labeled cells was calculated from the labeling index and the total number of cells, which was obtained separately. More than 500 cells were examined for each labeling index.

RESULTS

Establishment of the differentiation and dedifferentiation system. In order to analyze the differentiation and the dedifferentiation process quantitatively, we established the standard procedure as described in MATERIALS AND METHODS.

Results obtained with this procedure are shown in Figs. 1 and 2. The TPA-treated exponentially growing cells began to attach to the substratum in several minutes, and most of them had spread widely over the culture dish after 48 h, as earlier reports described (5, 7, 13) (Fig. 1A). Cell proliferation slowed down and the number of cells reached a maximum level on day 2 (Fig. 2A). From there on, the number of adherent, living cells decreased gradually to about half the initial count. By day 4 or 5 a great number of vesicles appeared in the cytoplasm of every cell, identifying them as mature macrophages (Fig. 1B). On day 8 or 9, most cells which has been firmly attached to the dish became round at almost

Fig. 3. Cell cycle. The cells were cultured as described in Fig. 2, fixed with 70% ethanol at 0, 8, 24 and 48 h on days 3 and 9, stained with ethidium bromide (0.01% in PBS) and analyzed with a cytofluorometer.
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The DNA synthesis was measured through $^3$H-TdR uptake, and results are shown in Fig. 2B. After treatment with TPA, the uptake of $^3$H-TdR decreased, reached its minimum on day 3 of the culturing period, and remained at a low level. From day 8 on, $^3$H-TdR uptake increased, which was followed by an increase in cell number. Finally, the rate of $^3$H-TdR uptake returned to that of exponentially growing cells (this value is shown as an asterisk on the ordinate of Fig. 2B).

Most cells dedifferentiating on day 8 can be traced back to well-differentiated cells on day 5 by comparing photographs of the same region of one dish every day. A few typical examples are shown in Figs. IB and IC.

**Cell analysis in the established system.** To describe the process of dedifferentiation, we analyzed some properties of cells treated by our standard procedure. The results of our cytofluorometric analysis are shown in Fig. 3. The retardation of the cell cycle was observed 8 h, and cell cycle progression almost stopped completely, 48 h after the addition of TPA. From day 2 or day 3 of incubation, the cells accumulated at the G0/G1 phase, and most cells remained at that phase for several days. On day 9, cells in their S to G2/M phase reappeared. These results correspond closely to the changes in cell number and DNA synthesis shown in Fig. 2.

Morphological features of these cells were those of differentiated macrophages, as shown in Fig. 1B. Other phenotypes characteristic of differentiated macrophages, i.e., the activity of Fc-receptor-mediated phagocytosis and superoxide production, were also analyzed. The changes in Fc-receptor-mediated phagocytosis during our standard procedure are shown in Fig. 4A. The percentage of positive cells increased from 0 to 80 percent in 3 days, reached its maximum value of 93.6% on day 6, and gradually decreased from there on.

Superoxide production was measured by means of NBT reduction (Fig. 4B). The percentage of NBT reducing cells showed changes similar to those in percentage of phagocytosis-positive cells: it increased, reached a maximum value on day 6, remained at that level for 3 days, and then decreased. It is noteworthy that the DNA synthesis of the dedifferentiating cell population began to increase on the 8th day and that these cells at the same time began to lose their activity of Fc-receptor-mediated phagocytosis (Fig. 2B, Fig. 4A). To examine which part of the cell population synthesized DNA, we BrdU-pulse-labeled the differentiated culture and detected DNA synthesizing cells by staining cells with FITC-conjugated anti-BrdU antibody.

When cells were labeled on the third day of incubation, only 6% (23 out of 354 counted cells) were stained, while with BrdU-pulse-labeling on the 7th day, about 57% (187 out of 327 counted cells) were stained (Fig. 5). The positive cells were distributed evenly on the dish and did not form colonies. This implies that DNA synthesis was initiated anew by the majority of cells during...
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Fig. 5.
differentiating at the time and not by a special nondifferentiated subpopulation.

Changes in the cell number (Fig. 2A) indicated that more than half of the TPA-treated cells were lost from the culture in the early part of this procedure. To investigate what kind of cells were lost, cells were labeled with \(^{3}H\)-Tdr (1 \(\mu Ci/ml\)) immediately before they were treated with TPA. They were then fixed and subjected to autoradiography after incubation for 2, 5 and 8 days. About half of the exponentially growing cells were labeled. The number of labeled cells increased at first and then decreased markedly, while that of nonlabeled cells remained fairly constant (Fig. 6). Changes in the cell number in this experiment coincided with those for our standard procedure (Fig. 2). This led us to believe that the possible toxic effects of radioisotopes did not affect our experimental results, although they might preferentially kill cells incorporating \(^{3}H\)-Tdr. To confirm our supposition, two more sets of experiments were carried out with different concentrations (0.1 \(\mu Ci/ml\) and 10 \(\mu Ci/ml\)) of \(^{3}H\)-Tdr. The number of grains per cell was markedly different, but the ratio of cells with grains to those without grains was virtually the same on each assessment day. This ruled out the possibility that cells were killed by \(^{3}H\)-Tdr-label in our experiments.

Dedifferentiated, floating cells were morphologically indistinguishable from those used as starting materials. We cultured these floating cells for several days and subjected a portion of them to a second TPA treatment. This differentiation of dedifferentiated cells revealed cells adhering to the dish and being widely spread (Fig. 7A). The cells also expressed functions characteristic of macrophages (Fig. 7B). Our culture system showed that fully differentiated cells started to dedifferentiate on the 8th day of incubation. The maintenance of the differentiated state and cell proliferation might therefore be inversely related.

**DISCUSSION**

On the basis of the time course of the increase in cell numbers and the doubling time of the U937 cell (about 19 h in exponential phase), we concluded that the cells that synthesized DNA proliferated in the latter half of the standard procedure were those that dedifferentiated in the system. Reinitiation of DNA synthesis and cell proliferation occurred about one day earlier when we used cover slips instead of plastic culture dishes (cf. Fig. 1 and Fig. 5). We demonstrated that dedifferentiated cells were sensitive to TPA and could differentiate into macrophages again (Fig. 7A, B). This showed that they were not a special subpopulation that escaped TPA treatment.

Our results and the standard procedure adopted in this study eliminate the assumptions that cells resistant or insensitive to TPA persist in the culture and that reappearing floating cells are due to the selection of a specific subpopulation of U937. On the contrary, it is to be concluded that the simultaneous disappearance of differentiated cells and the reappearance of proliferating cells were due to the reversion of the differentiation process. TPA treatment seems to generate a signal for the U937 cell to differentiate, and cells regain their proliferating activity after its effect has disappeared. There were, however, some dying cells that were lost from the culture in the first half of the procedure. It is generally accepted that the terminal cell differentiation occurs after the cell division. TPA-treated U937 cells began to adhere to the substratum after several minutes and changed their morphology after several hours. Our investigations revealed that most of the cells lost from the
Dedifferentiation of U937 culture were synthesizing DNA at the time of TPA treatment (Fig. 6). The addition of TPA apparently triggered the differentiation process before the ongoing DNA synthesis could be completed, thus destroying the integrity of the cell.

We reported previously that the induction of the v-mos gene into U937 cells caused macrophage differentiation and that the maintenance of the differentiated state required continued expression of the v-mos gene (8). This implies that the up-regulation of protein kinase C, which is the target of TPA, is not the only factor in the reversion of differentiation in U937 cells. Since a lymphocyte-conditioned medium and β-interferon induce macrophage differentiation of U937 (5, 7), it is possible to ascribe the acceleration of dedifferentiation to the frequent medium renewal, which would have deprived the culture medium of factors produced and secreted by differentiated U937 cells which are necessary for the maintenance of the differentiated state.

A human promyelocytic leukemia cell line, HL60, was reported to differentiate into macrophages when treated with TPA (13). Although observed for only 2 to 4 days, the phenomenon was described as irreversible and independent of the continuous presence of TPA. Applying the standard procedure we developed for this study, we treated HL60 cells with TPA and confirmed that they did not dedifferentiate even after prolonged culture (data not shown). It is not known why U937 dedifferentiates whereas HL60 does not. One possible reason is that the two cell lines differ in their oncogenesis, as the N-ras-proto-oncogene of HL60 is mutated and its genome transforms NIH3T3 (12), which the genome of U937 does not do (6).

Further analysis of the intracellular process of dedifferentiation will reveal the mechanism that maintains the differentiated state and should facilitate leukemia therapy. The culture system we developed and described in this study should prove useful for these investigations.

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REFERENCES


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