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Cofactors in *In Vitro* Induction of Apoptosis in HL60 Cells by All-*trans* Retinoic Acid (ATRA)

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ABSTRACT. The aim of this study was to determine the culture conditions that could modulate the induction of apoptosis by all-*trans* retinoic acid (ATRA). Cell viability was evaluated by trypan blue test, differentiation by nitro blue tetrazolium test, and apoptosis by morphological analysis. ATRA induced apoptosis in HL60 cells only when more than 100,000 cells/mL were seeded, while differentiation was induced regardless of the seeded cell concentration. Reduction in the concentration of foetal calf serum or glutamine in the medium led to a weak increase in apoptosis. In contrast, a dramatic enhancement of apoptosis occurred when the culture medium was supplemented with glucose or when the culture pH was decreased. These characteristics were independent of the mechanism of action of ATRA, but the action of glucose could be of significance in diabetic patients. An exchange of supernatants after 3 days of culture showed that supernatants from control cultures seeded at high cell density were better apoptosis inducers than supernatants from cultures treated with ATRA, but seeded at low cell density. Factor(s) in this supernatant which induced apoptosis was (were) removed by ultrafiltration. In conclusion, our results showed that ATRA alone cannot induce apoptosis, but can do so in conjunction with cofactors. The depletion of some components of the medium and the appearance of secreted macromolecule(s) could be cofactor(s) in the induction of apoptosis. BIOCHEM PHARMACOL **55**;2:177–184, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. apoptosis; all trans retinoic acid; ATRA; glucose

It is well known that all-*trans* retinoic acid (ATRA)§ induces differentiation in HL60 cells [1–6], although some authors have reported that differentiation seemed to depend on the culture conditions [7, 8]. On the contrary, few data refer to apoptosis induction by ATRA. Furthermore, when it was reported, the apoptosis rate was very low, to a maximum of approximately 15% [2, 9], when a high proportion of HL60 cells can enter apoptosis under the effect of anticancer drugs [9–12].

The induction of apoptosis by ATRA could account for its efficacy in the chemotherapy of patients with acute promyelocytic leukaemia [13]. For this reason, *in vitro* induction of apoptosis may very well be of interest, since it could contribute to our understanding of its mechanism of action and thereby improve its efficacy. However, in our studies ATRA was able to induce differentiation but not apoptosis. Indeed, neither Martin et al. [2] nor McCarthy et al. [9] gave the essential information concerning the induction of apoptosis, i.e., the cell concentration at seeding. This could indicate, then, that the activity of ATRA on apoptosis is not as direct as for anticancer drugs [14]. In order to clarify this phenomenon, we studied the culture conditions which might interfere with the induction of apoptosis, such as cell, foetal calf serum (FCS), glutamine and glucose concentrations as well as pH value. An inducer of apoptosis was sought for in the culture supernatant. In passing, we noticed that a high glucose concentration in the culture medium was a potent inducer of apoptosis, which could explain some long-term problems in diabetes mellitus.

MATERIALS AND METHODS

Chemicals

PBS, pH 7.4 and RPMI 1640 (pH 7.3) were obtained from Life Technologies. FCS (Life Technologies) and glutamine (Sigma) were added to the culture medium. May-Grünwald and Giemsa staining reagents were obtained from Merck and trypan blue from Prolabo. The other chemicals were from Sigma. Unless otherwise stated, ATRA (Lab. Hoffmann-La Roche) was used at a concentration of 1 μM for the whole period of incubation.

Cell Culture

HL60 cells were kindly provided by Chomienne. Unless otherwise stated, they were grown in RPMI 1640 contain-

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§ *Abbreviations:* ATRA, all-*trans* retinoic acid; FCS, foetal calf serum; NBT, nitro blue tetrazolium; NBT^+, NBT positive cells; TB^+, trypan blue positive.

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ing 10 mM glucose and supplemented with 15% FCS and 2 mM glutamine. Cells were maintained at 37° in a 5% CO_2/95% air atmosphere. Exponentially growing cells were pelleted by centrifugation for 5 min at 170 × *g* and seeded at the stated concentration in complete medium containing the indicated glucose and drug concentrations. In some experiments, aliquots were harvested at the stated times in order to count cells and to determine the glucose concentration in the medium.

For supernatant exchange experiments, cell cultures were centrifuged at 170 × *g* for 5 min on day 3 of incubation, and the cell pellets were reincubated for the subsequent 2 days with supernatants of a separate culture. For ultrafiltration experiments, the same schedule was used, and the components with a molecular mass greater than 10,000 were removed by ultrafiltration (Centriprep).

Cells were counted using a Malassez hematocytometer. The effect on cell growth was expressed as the percentage of the doubling number per 24 hr of control cells. The glucose level in the culture medium was determined using glucose GOD PAP reagents (ref #1 448 676, Boehringer Mannheim).

For the study of the pH effect on apoptosis induction, the former was verified twice a day and eventually adjusted to *ca.* pH 7.0 and 7.8 using HCl 1N and NaOH 1N, respectively. The pH of control cells was verified only.

Evaluation of Differentiation

The nitro blue tetrazolium (NBT) reduction test was adapted from Breitman [3]: 200,000 cells were washed twice with PBS and incubated for 30 min at 37° in the dark with 200 μL NBT solution containing 1 mM NBT, 167 nM 12-O-tetradecanoylphorbol 13-acetate and 8% FCS in RPMI 1640. Differentiated HL60 cells reduce NBT to produce blue-black, cell-associated, nitro blue diformazan deposits. The number of cells able to reduce NBT (NBT^+) were counted with an optical microscope using a Malassez hematocytometer. Results are expressed as a percentage of living cells.

Evaluation of Cell Death

Primary and secondary (postapoptotic) necrotic cells were assessed as trypan blue positive (TB^+) cells. For apoptosis evaluation, cells were centrifuged at 30 × *g* for 5 min on a Cyto-Tek slide cytocentrifuge (Miles Scientific) and stained by the May-Grünwald Giemsa method. Apoptotic cells were characterised by a condensation or fragmentation of the nucleus as assessed using an optical microscope. More than 300 cells were counted per slide, with the results expressed as percent. As the reproducibility of the percentage of apoptotic cells is poor, the results of a typical experiment are shown.

Transmission Electron Microscopy

Cells were fixed in 2.5% glutaraldehyde in PBS, pH 7.2 for 1 hr at room temperature. They were washed in water and pelleted in a bovine serum albumin (BSA) 7%-glutaraldehyde 25% (50/50 v/v) mixture according to Shands [15]. They were then processed for acetylation specific for ribonucleoproteins, according to Wassef [16]. Embedding was done into LR White. Ultrathin sections were counterstained with uranyl and lead. Observations were made using a Hitachi H300 electron microscope at 75 kv.

RESULTS

In control cultures, the cell concentration at day 5 did not exceed 2 million cells/mL, regardless of glucose and seeded cell concentrations. With the lower cell concentrations, i.e., 25,000 and 50,000 cells/mL (Fig. 1A and B), cell growth appeared exponential, but was not exponential for the highest cell concentrations of 200,000 and 400,000 cells/mL (Fig. 1C). For 100,000 cells/mL, the pattern of cell growth was intermediate (Fig. 1B). In conclusion, increasing the initial cell concentration induced a reduction of cell growth in control cultures. As a consequence, inhibition of cell growth by 1 μM ATRA was more difficult to detect at the higher cell concentrations.

ATRA did not induce apoptosis at low initial cell concentrations; apoptosis occurred only when more than 100,000 cells/mL were seeded (Fig. 2). In the presence of 1 μM ATRA, the proportion of cells reducing NBT reached a plateau at approximately 80% NBT^+ cells, regardless of the number of seeded cells (data not shown).

For a seeding of 200,000 cells/mL, ATRA at a concentration of 0.1, 0.3 and 1 μM slightly decreased the cell growth in a dose-related manner. It induced more than 80% NBT^+ cells whatever the ATRA concentration, while apoptosis occurred only at a concentration of 1 μM (Fig. 3).

Control cells (Fig. 4A) showed a wide nucleus whose components consisted of clumps of chromatin scattered within nucleoplasm or underlining the nuclear envelope. One or two nucleoli appeared roundish, with typical perinucleolar chromatin and a wide fibrillar centre joined to a dense fibrillar component. The granular component appeared as a ring around the fibrillar center. When treated with ATRA 1 μM for 5 days, some cells showed only slight modification of the nucleus which concerned the chromatin condensation, and clumps were few and smaller (Fig. 4B). Other cells showed typical apoptosis configuration of the nucleus, which was fragmented into several electron opaque structures (Fig. 4C). Moreover, cytoplasm exhibited numerous vesicles. Early steps of apoptosis showed that all HL60 cells observed had already undergone differentiation, as their nuclei were highly gulfed. Some apoptotic cells lost their plasma membrane, thus corresponding to postapoptotic necrosis [17]; only infrequent primary necroses were visible.

This sensitivity to apoptosis with high cell concentration could be attributed to the disappearance or appearance of

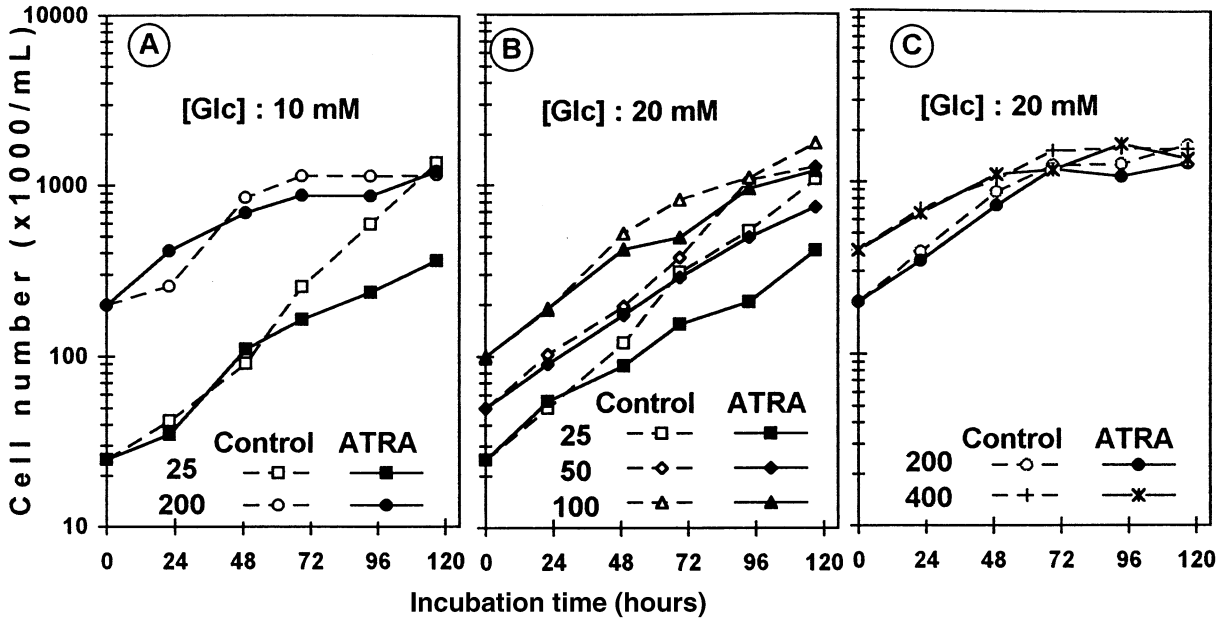

FIG. 1. Cell growth during 5-day incubations in medium containing 10 or 20 mM of glucose in control and ATRA-treated cultures. Cell concentration (×1000/mL) at seeding is indicated.

compound(s) in the culture medium. When the concentrations of FCS and glutamine were reduced independently, apoptosis seemed to increase slightly (Table 1).

Since glucose concentration decreased very quickly in the culture medium when high cell concentrations were used, it was increased to 20 mM for some experiments. Under these conditions, the medium was not depleted of glucose at the end of the incubation, with the final concentrations of glucose between 18 and 6 mM. Apoptosis was visible mainly in cultures where the residual glucose concentration was less than 14 mM of glucose, i.e., at higher cell concentrations. With the 20 mM glucose medium, apoptosis was more pronounced in control than in treated cultures except for the lowest cell concentration, where apoptosis was not visible (Fig. 5). With 200,000 and 400,000 cells/mL at seeding, apoptosis decreased after days 4 and 3, respectively, in control cultures, but increased during one additional day in treated cultures (Fig. 5C). The decrease in apoptosis was followed by a dramatic increase in the proportion of TB^+ cells (Fig. 6C). Similarly, with the highest cell concentration, apoptosis in ATRA-treated cultures increased up to a maximum at day 4 and then decreased (Fig. 5C), while the TB^+ cell proportion subsequently increased (Fig. 6C). In all kinetic experiments, the TB^+ cell proportion was elevated only after an initial sharp increase in apoptosis (Fig. 6).

In another series of experiments, the medium pH was either not modified, or kept above 7.3 or below 7.0. It appeared in this case that it did not return to a normal value after the first day, even decreasing below 6.0 during the fourth day (Fig. 7A). Acidic conditions greatly favoured apoptosis and clearly affected cell growth, whereas alkaline

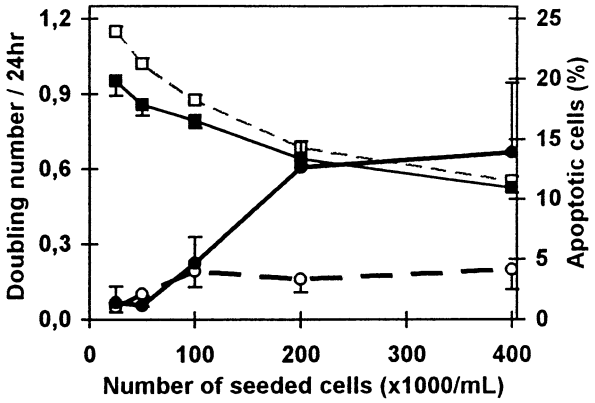

FIG. 2. Effect of cell concentration at culture onset on cell growth assessed by the doubling number (square) and the proportion of apoptotic cells (circle) after a 5-day incubation. Control cells (open symbols) and 1 μM ATRA-treated cells (closed symbols). Values are means ± SE of 4 experiments.

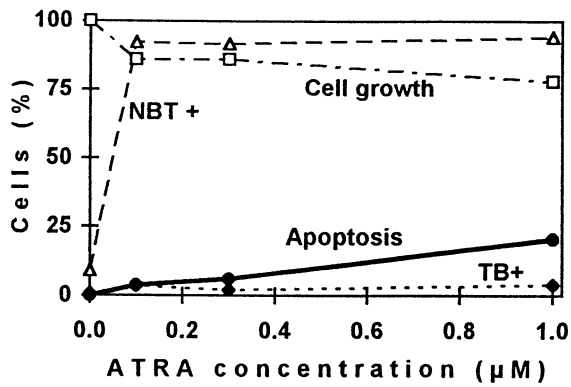

FIG. 3. Effect of increasing ATRA concentration on cell growth expressed as percentage of the doubling number of control cells (square), percentage of NBT^+ cells (triangle), apoptotic (circle) and TB^+ cells (diamond) after a 5-day incubation (seeding of 200,000 cells/mL).

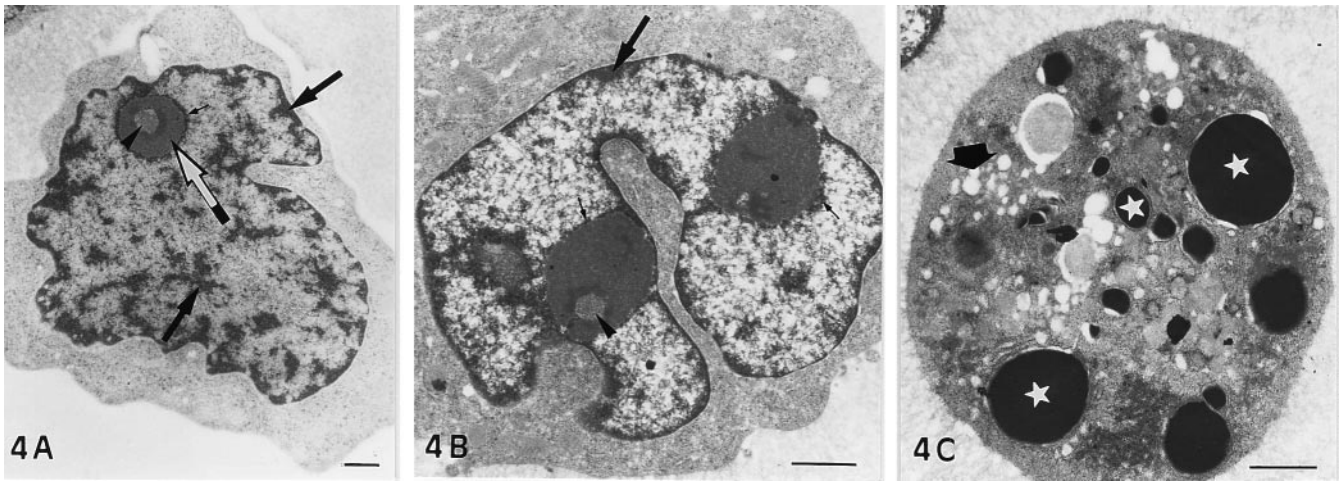

FIG. 4. A) Control HL60 cell; ×5000; bar = 1 μm. The nucleus exhibited clumps of chromatin (➡), either scattered within nucleoplasm or joined to the nuclear envelope. Nucleolus showed perinucleolar chromatin (→), fibrillar center (▶) surrounded by dense fibrillar component. Granular component (⇒). B–C) HL60 cells treated by 1 μM ATRA; ×9000; bar = 1 μm. B) Cell with minor modification compared to control cells, concerning the fewer chromatin clumps within nucleoplasm. C) Cell with several apoptotic bodies (★) and cytoplasmic vesicles (↑).

conditions had no effect as compared to nonmodified medium (Fig. 7B,C).

In a further series of experiments, the supernatant of cultures containing 10 or 20 mM glucose and incubated for 3 days was exchanged with cells incubated under different conditions for a subsequent 2-day incubation. The supernatant of control cultures seeded at 200,000 cells/mL induced a higher rate of apoptosis than the supernatant of cultures seeded at 25,000 cells/mL with ATRA, whatever the cells used for the second period of incubation and regardless of the glucose concentration in the medium (Table 2).

In some experiments, the supernatants of cultures were cleared of species of molecular weight greater than 10,000 by filtration. This filtered medium was then added to the corresponding cells for the second period of incubation. The potency of supernatant from cultures including ATRA and seeded at 200,000 cells/mL in inducing apoptosis was reduced by 46 and 60% for 10 and 20 mM glucose medium, respectively. The addition of fresh FCS did not restore the potency of this medium in inducing apoptosis. In control cultures seeded at 25,000 and 200,000 cells/mL with 20 mM glucose, the proportion of apoptotic cells was reduced from 11.1 and 35.0% in this series of experiments to 2.7 and 2.4% respectively, indicating that filtration almost entirely removed the ability to induce apoptosis.

DISCUSSION

It is clear from this report that under normal culture conditions, i.e., when cell growth is exponential, ATRA induces differentiation of HL60 cells, but does not induce apoptosis. Induction of apoptosis requires a minimum threshold cell concentration. Other reports found no induction of apoptosis by ATRA in HL60 cells, which could be the result of culture conditions [18]. Similarly, it has been reported that 9-*cis* retinoic acid and aclarubicin need specific culture conditions in order to exert a maximal induction of apoptosis in HL60 cells [11, 19].

These results suggest that ATRA requires cofactors, but their mechanisms of action are not clear. It has been reported, for example, that ATRA inhibits the induction of apoptosis by anticancer drugs such as etoposide, camptothecin and actinomycin-D [9]. It has also been reported that the effect of ATRA was potentiated by topoisomerase inhibitors [4]. Under our conditions, it is not easy to determine if the effect of ATRA was potentiated by the culture conditions, or on the contrary, if a particular

TABLE 1. Percentage of apoptosis in cultures where FCS or glutamine concentrations were modified (10 mM glucose)

	FCS concentration (%)						Glutamine concentration (mM)		
	1	2.5	5	10	15	20	2	4	6
25,000 cells/mL at seeding									
Control	1.1	1.3	1.5	2.8	3.0				
ATRA	4.9	6.0	1.8	0.9	0.6				
200,000 cells/mL at seeding									
Control	*	*	1.0	3.9	4.4	2.1	17.7	8.6	4.6
ATRA	*	*	13.0	5.3	9.5	5.3	20.7	14.0	11.4

* Too much necrosis to evaluate apoptosis.

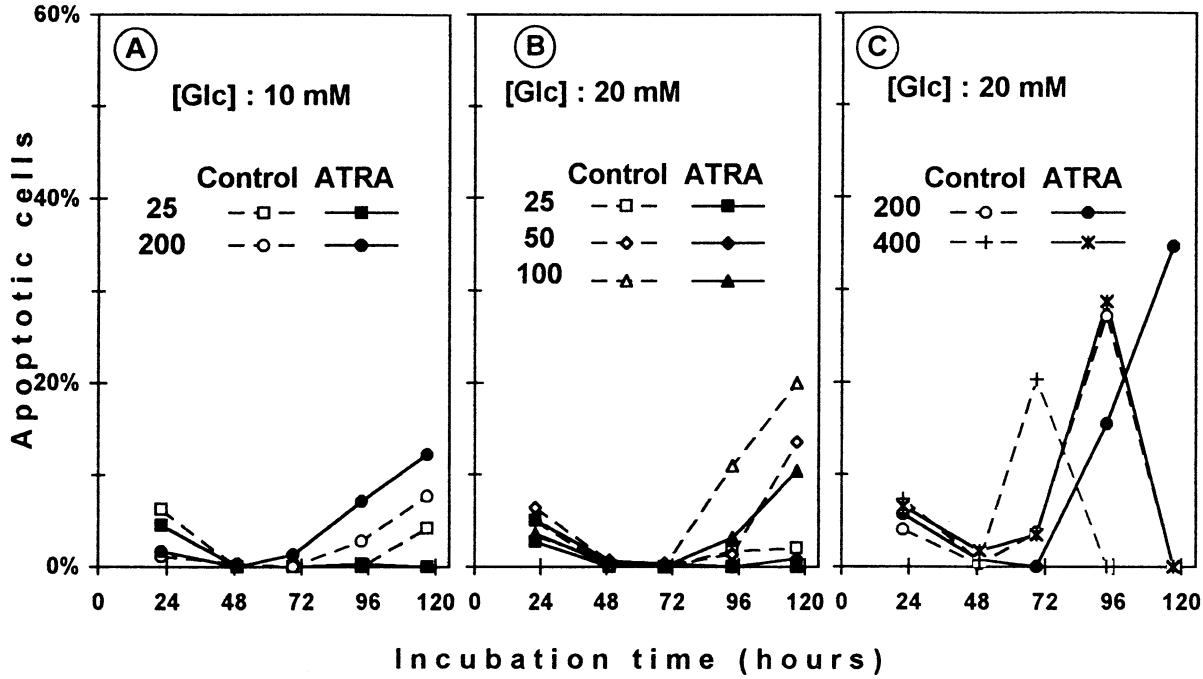

FIG. 5. Kinetics of apoptosis during 5-day incubations in medium containing 10 or 20 mM of glucose in control and ATRA-treated cultures. Cell concentration (×1000/mL) at seeding is indicated.

property of ATRA potentiated the effect of the culture conditions. It was determined previously that ATRA decreases the cellular concentration of *bcl2* [9, 20], an inhibitor of apoptosis [21]. The induction of apoptosis could also be a consequence of the cumulative effect of the culture conditions, ATRA and the cytokines secreted under the induction of ATRA [5].

It must be pointed out that under all conditions (with the exception of high glucose concentration), apoptosis induction was not considerable. Furthermore, the experiments were not entirely reproducible, although the results were always along the same lines. This great variability could be explained by the intervention of a great number of culture parameters in apoptosis induction. Although we were very careful in regard to cell culture, it is possible that we did not reproduce one or several parameters from one experiment to another.

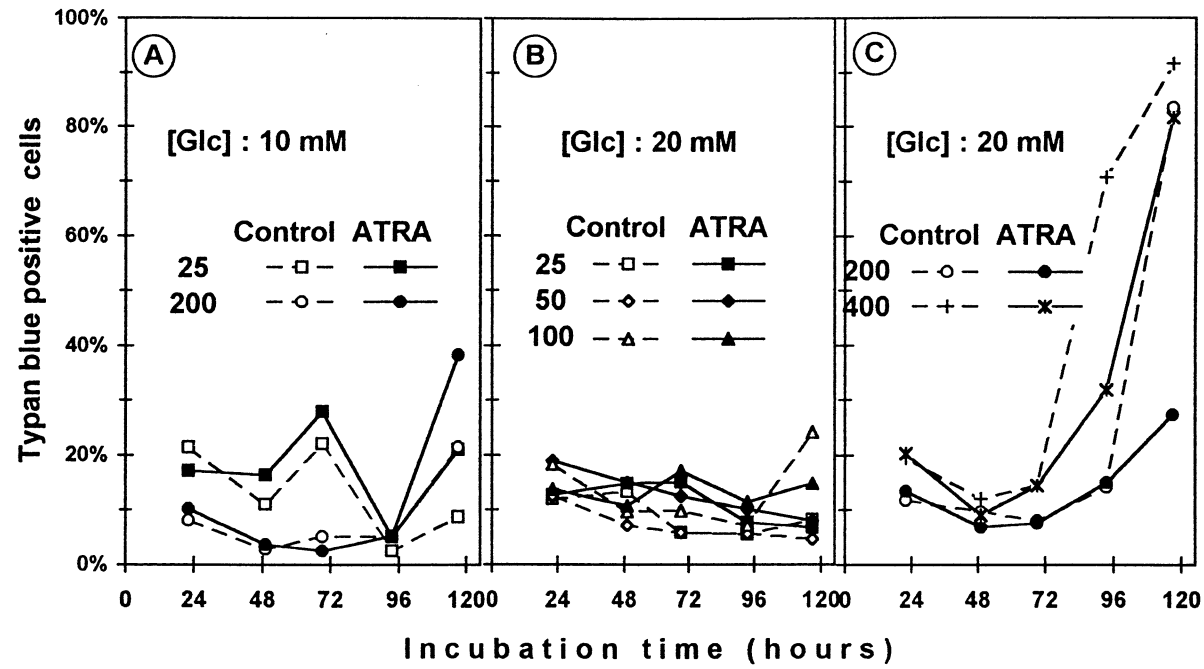

FIG. 6. Kinetics of TB+ cell appearance during 5-day incubations in medium containing 10 or 20 mM of glucose in control and ATRA-treated cultures. Cell concentration (×1000/mL) at seeding is indicated.

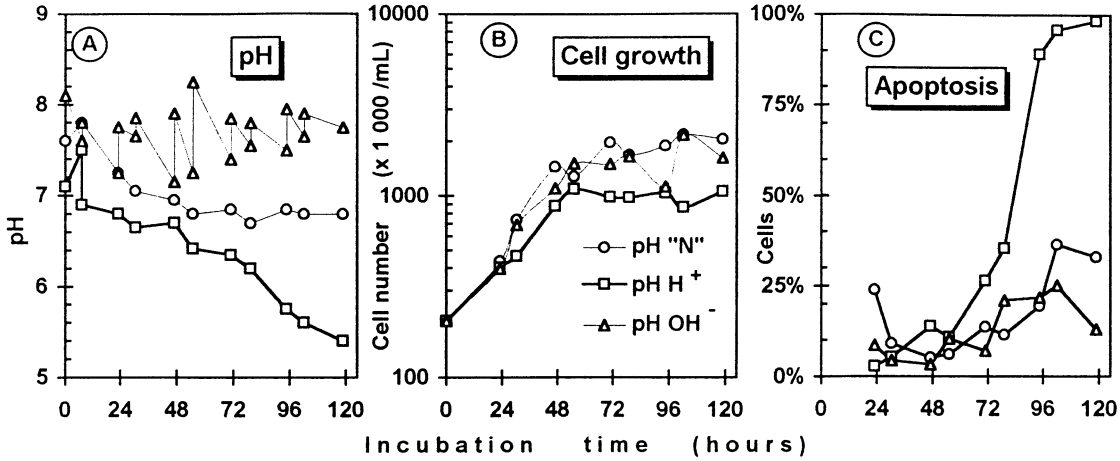

FIG. 7. Kinetics of pH (A), cell growth (B), apoptosis (C) of nontreated cultures during 5-day incubations in nonmodified (N, control), acidic (H^+) or alkaline (OH^-) medium (seeding of 200,000 cells/mL).

Induction of apoptosis was dose-related, the dose required being superior to that producing differentiation. In Fig. 3, it can be seen that induction of differentiation was maximum with all ATRA concentrations tested, whereas apoptosis induction was perceptible only at the highest ATRA concentration tested. Thus, it can be concluded that, as for aclarubicin, the induction of differentiation by ATRA is independent of the induction of apoptosis [11]. It has been reported that apoptosis is not always a simple consequence of differentiation and that differentiation and apoptosis could be regulated independently in myeloid cells [6, 22]. As observed in our results, all apoptotic cells seemed to be differentiated cells. As the HL60 cells did not directly respond to ATRA under these conditions, the modulation of apoptosis could correspond to the modulation of any step in the cell maturation leading to apoptosis.

Under optimal conditions for apoptosis, kinetic studies showed that the proportion of apoptotic cells increased sharply at day 3, 4 or 5, depending on the culture conditions (Fig. 5). This increase was followed by a decrease, during which the proportion of TB^+ cells increased sharply (Fig. 6). It can be hypothesized that cells with an apoptotic morphology evolve to a postapoptotic morphology, i.e., with secondary necrotic cells incorporating trypan blue dye [17]. These kinetic studies are important in designing studies of apoptotic induction. If an evaluation were based on data from day 5 only, it could be concluded from Fig. 5C, for example, that with 200,000 cells at seeding, apoptosis was higher in ATRA-treated cultures than in control. A kinetic study would indicate that with 20 mM of glucose, apoptosis would occur earlier in control than in treated cultures. In these controls, the rate of apoptosis may be higher than in treated cultures, since in our experiments the true peak would not be detected, as it occurs at *ca.* 96 h (Fig. 5C).

The induction of apoptosis by ATRA requires cofactor(s), which may be negative (consumed) and/or positive (secreted by cells). The more cells there are in the culture, the faster nutrients will be depleted from the medium, perhaps even completely. The consequences of reduced FCS and glutamine concentrations were investigated. It is known that removal of FCS induces apoptosis [23], but in our experiments, the amount of FCS was sufficient in all cultures to avoid apoptosis in control incubations. In treated cultures, however, reduced FCS enhanced apoptosis. The fact that ATRA is bound to plasma proteins could explain why low FCS concentrations, i.e., increased free ATRA, were correlated with higher apoptosis inductions, and high FCS concentrations, i.e., decreased free ATRA, were correlated with lower apoptosis inductions.

When the concentration of glutamine was reduced, a slight increase in apoptosis in control and treated cultures was seen. Depletion of glucose might also be partly responsible for the induction of apoptosis. The glucose concentration was 10 mM in the medium; when more than 100,000 cells were seeded per mL, almost no glucose remained at the end of the incubation. Surprisingly, with an initial concentra-

TABLE 2. Percentage of apoptosis in 5-day cultures where the supernatant was exchanged or not at Day 3

	10 mM glucose			20 mM glucose	
Supernatant	No cell exchange	ATRA-25,000 cells	ATRA-200,000 cells	No cell exchange	ATRA-25,000 cells
Control-25,000	1.3	—	5.3	11.1	0.7
Control-200,000	4.8	6.0	11.3	35.0	10.8
ATRA-25,000	1.5	1.5	9.8	0.3	0.3
ATRA-200,000	19.1	21.3	19.1	31.7	30.0

tion of 20 mM glucose, there was an increase in apoptosis. This increase occurred earlier and was slightly stronger in control than in ATRA treated cultures. Under these conditions, ATRA appears to act either as a protectant against apoptosis or to at least delay its onset (Fig. 5).

The induction of apoptosis by high a glucose concentration is very interesting. The same phenomenon was recently reported with endothelial cells [24], but it was noted that certain cells were not sensitive to glucose. This phenomenon could account for damage to blood vessels in diabetic patients.

Our culture conditions used in order to induce consistent levels of apoptosis appeared to correspond to an acidification of the medium throughout the incubation time. Since an intracellular acidification could be responsible for apoptosis [25], different pH conditions were tested. Indeed, an acidic medium increased apoptosis after 3 days of incubation, when the medium pH was below 6.5, in accord with Park et al. [25]. This could be due to the activation of the endonuclease DNase II [12].

It appeared from our results that the depletion of FCS, glutamine or glucose could not account for the degree of apoptosis induced, whereas the effect of the pH decrease could be more controversial. It is possible that a factor secreted by cells was also involved. Incubations with an exchange of supernatants showed that the supernatant of control cultures seeded at 200,000 cells/mL induced more apoptosis than the supernatant from ATRA-treated cultures seeded at 25,000 cells/mL. The composition of the medium had a greater influence than the presence of ATRA. Ultrafiltration led to a reduction in apoptosis, suggesting that component(s) removed from the supernatant might act as cofactors in the induction of apoptosis. This effect was not reversed by the addition of FCS, indicating that the eliminated component may be secreted by cells in culture at high density. These component(s) is (are) of molecular weight greater than 10,000. This is in accordance with the reported secretion of cytokines by HL60 cells, which play a significant role after two days of incubation [5], and is compatible with the observed delay in apoptosis.

The induction of apoptosis by ATRA could account for its efficacy in the chemotherapy of patients with acute promyelocytic leukaemia. As we have pointed out in this paper, however, this induction of apoptosis *in vitro* requires particular conditions. The most frequent particular condition found in the literature seemed to be a high cell concentration at seeding, although no author mentioned that this condition of apoptosis induction was particular. The requirement of a high cell concentration at seeding could correspond to the exhaustion of some components of the medium and to the secretion of macromolecular cofactors by cells.

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