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Induction of embryonal carcinoma cell differentiation by deferoxamine, a potent therapeutic iron chelator

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Abstract

We investigated the effects of deferoxamine on the differentiation of embryonal carcinoma F9 cells. Deferoxamine, a widely used therapeutic agent for thalassemia and iron overload, was found to induce F9 cell differentiation and to have some unique characteristics compared with other chelators, hinokitiol and dithizone, which were previously reported to induce differentiation of these cells. This hydrophilic agent induced reversible differentiation as did sodium butyrate, whereas other chelators did not. However, morphological features of the cells after deferoxamine-induced differentiation were similar to those of cells incubated with the other chelators. The differentiation-inducing activity of deferoxamine was abolished by preincubation with Fe^{3+} ions, similarly to the other chelators examined. Moreover, cell proliferation was inhibited by treatment with this agent, and the numbers of cells in the colonies were reduced by apoptosis. Based on these results, we conclude that deferoxamine induces differentiation and apoptosis of F9 cells via chelation of extracellular and/or intracellular Fe^{3+} ions.

Keywords: F9 cell; Deferoxamine; Chelator; Differentiation; Apoptosis

1. Introduction

Mouse F9 cells, teratocarcinoma-derived embryonal carcinoma cells cloned by Bernstein [1], are pluripotent cells with a very low rate of spontaneous differentiation. These cells have been reported to differentiate into embryonic endoderm cells following treatment with retinoic acid, sodium butyrate, antibiotics and potent iron chelators such as hinokitiol and dithizone [2–6]. The phenotypes of differentiated and undifferentiated F9 cells are different both morphologically and biochemically. For example, cell shape changes from spherical to polygonal with differentiation [2], and differentiated cells express extracellular matrix proteins such as plasminogen activator (PA), type IV collagen and laminin [7] accompanied by reduction in the level of expression of stage specific embryonic antigen-1 [8]. Moreover, it was recently reported that the transcription of several genes is activated during the differentiation process in F9 cells [9–11]. These changes are very similar to those occurring in the early stages of normal embryo-

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genesis, and thus they are a good model for studying both embryogenesis and mechanisms of differentiation [12].

We reported previously that the potent iron chelators tropolone, hinokitiol and dithizone induced the differentiation of F9 cells [5,6], and suggested that their abilities to chelate iron ions might be responsible for their biological activity. To clarify the mechanism of induction by chelators, we further investigated the differentiation-inducing activity of deferoxamine, an iron chelator used therapeutically. This agent was originally obtained from *Streptomyces pilosus* by Koberle in 1964 [13] and it has been reported to bind to an equimolar amount of ferric ion to form a stable complex [14]. Although deferoxamine can bind other metal ions such as copper, cobalt and zinc, its binding to ferric ion is the most stable. The affinity of deferoxamine to iron ions is higher than those of other chelators, e.g. ethylenediamine tetraacetic acid (EDTA) and diethylenetriamine pentaacetic acid [15]. Thus, deferoxamine has been widely used clinically in treatment of thalassemia and iron overload [15–18]. Deferoxamine was reported to block the cell cycle reversibly at the G1-S border [19]. It was also suggested that deferoxamine is an effective agent for cancer therapy, especially for treatment of neuroblastoma, because it is an inhibitor of DNA synthesis [20–22]. In a study using the human leukemia cell lines K562 and Daudi cells, the inhibition of DNA synthesis by this agent was shown to be due to its ability to block ribonucleotide reductase which participates in the synthesis of deoxyribonucleotides [23].

We found that deferoxamine has differentiation- and apoptosis-inducing activities in F9 cells, and also assessed its unique induction mechanism as compared with those of other chelators.

2. Materials and methods

2.1. Materials

Deferoxamine was purchased in mesylated form from Sigma Chemical Co. (St. Louis, MO, USA). Dithizone and ferric chloride were from Nacalai Tesque (Kyoto, Japan). DNA 100 bp ladder marker was from Pharmacia Biotech (Piscataway, NH, USA). All other reagents used were of analytical grade.

2.2. Cell culture

F9 cells were provided by Dr. Yoshitake Nishimune (Research Institute for Microbial Diseases, Osaka University). Stock cultures of F9 cells were grown on tissue culture-grade dishes (Iwaki Glass, Japan) in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Life Tech, Gaithersburg, MD, USA), 5 mM L-glutamine and 1 mM sodium pyruvate at 37°C in a humidified atmosphere of 5% CO_2-air. For experimental cultivation, the cells in stock cultures were detached from the surface of dishes by 0.2% trypsin-0.02% EDTA in phosphate buffered saline (PBS) at 37°C for 5 min, washed, and resuspended in MEM. Then, the cells were seeded at an appropriate cell density in dishes.

2.3. Assay of PA production

The assay method for determining PA-producing colonies was described elsewhere [24,25]. Briefly, cells were seeded at about 120 cells per 35 mm gelatin-coated dish (Nunclon, Denmark) and cultured for 3.5 days to allow proliferation and colonization. Then, the culture medium was changed to fresh MEM containing chelators. After treatment, colonies were counted macroscopically, washed with PBS and further incubated with 1 ml of prewarmed (43°C) agar-overlay solution containing 0.75% agar, 2.5% skimmed milk (Difco, Detroit, MI, USA) and 0.28 CU bovine plasminogen (Daiichi Pure Chemicals Co., Japan) in MEM. The numbers of PA-producing colonies were estimated by counting clear caseinolytic zones in the opaque layer. PA production rate was calculated for the percentage of the number of plaques relative to the total number of colonies in each dish.

2.4. Assay of cell proliferation

Cell proliferation was assayed by counting the numbers of cells. F9 cells (1×10^5) suspended in MEM were seeded into 6 cm dishes. After 48 h cultivation, medium was changed to fresh MEM con-

taining deferoxamine, and the cells were cultured for further 24 h. The number of cells was counted using a hematocytometer after detachment by trypsin-EDTA at the indicated times.

2.5. Detection of DNA fragmentation

Detection of DNA fragmentation as an indicator of apoptosis was performed by the method described by Ramachandra and Studzinski [26]. Briefly, total DNAs from deferoxamine-treated and non-treated cells were electrophoresed in 2% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

3. Results

3.1. Differentiation-inducing activity of deferoxamine

As shown in Fig. 1A, colonized F9 cells were treated at various concentrations of deferoxamine for 24 h. PA production rate, which indicates the percentage of differentiated colonies, was increased by this treatment in a dose-dependent manner. Maximum activity was observed at the concentration of 100 μg/ml (152 μM), and most of the colonies were differentiated at this concentration. At 5.0 μg/ml (7.6 μM), however, deferoxamine did not induce differentiation of F9 cells. This activity was comparable to that of hinokitiol at 10 μg/ml (60.9 μM) [5]. The production rate of PA was also dependent on the duration of treatment (Fig. 1B). A marked increase in PA production was observed after treatment with deferoxamine (100 μg/ml) for 8 h, and further enhancement was observed up to 24 h in a time-dependent manner.

3.2. Morphological changes

After 24 h treatment of F9 cells with deferoxamine at 100 μg/ml, the cells changed to a polygonal shape typical of differentiated F9 cells (Fig. 2B) [2,5]. The morphology of F9 cells treated with deferoxamine for 24 h was markedly different from that of undifferentiated control cells (Fig. 2A).

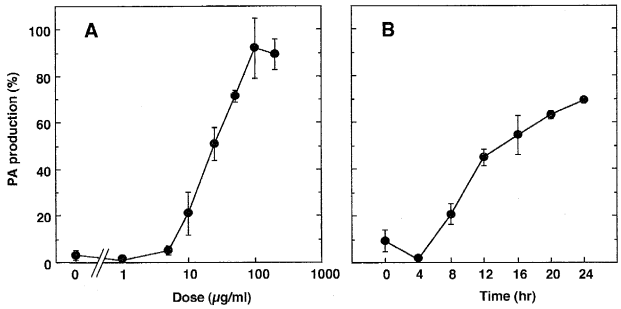

Fig. 1. Effects of deferoxamine on PA production in F9 cells. PA production rate (%) was estimated by the agar-overlay method in which the formation of caseinolytic plaques was detected. (A) Dose-response curve of deferoxamine on PA production. F9 colonies were treated with various concentrations of deferoxamine for 24 h. (B) Time-course of PA production. F9 colonies were treated with 100 μg/ml deferoxamine for the indicated times. All data represent means $\pm$ S.D. of triplicate cultures.

3.3. Inhibitory effect of metal ions on deferoxamine-induced differentiation of F9 cells

We previously reported that differentiation-inducing activities of the chelators, tropolone, hinokitiol, and dithizone, were diminished by treatment with Fe^{3+} ions prior to addition to cultures of F9 cells [6]. To assess the role of iron chelation in deferoxamine-induced differentiation, we investigated the effect of preincubation with Fe^{3+} ions (Fig. 3). Pretreatment of 50 μg/ml (76 μM) deferoxamine with 40 μM and 60 μM Fe^{3+} decreased PA production rates of F9 cells to 76.4% and 41.8%, respectively, of those of non-pretreated deferoxamine-treated cells. Preincubation with more than 80 μM Fe^{3+} blocked the ability of deferoxamine to induce differentiation of F9 cells.

3.4. Reversibility of differentiation of F9 cells by deferoxamine

It was reported that the differentiation of F9 cells treated with retinoic acid and hinokitiol was irreversible [5,27], while differentiation with sodium butyrate was reversible [25]. We investigated the reversibility of differentiation in cells treated with deferoxamine and dithizone (Fig. 4). The colonized F9 cells were differentiated by treatment with 100 μg/ml deferoxamine and 10 μg/ml dithizone for 24

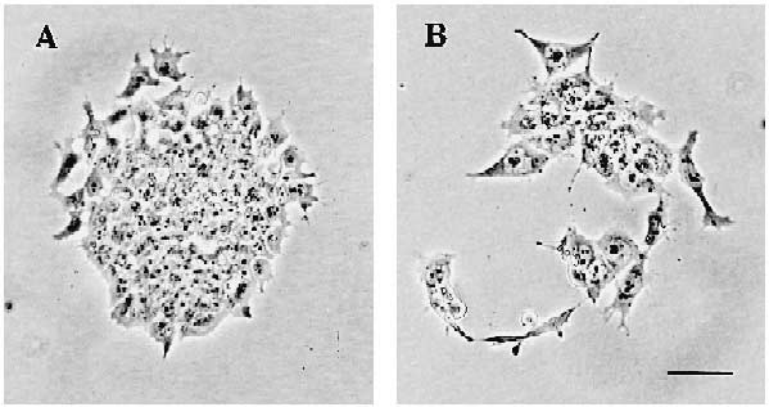

Fig. 2. Morphological changes of F9 cells treated with deferoxamine. F9 cells were plated on gelatin-coated dishes and treated with (B) or without (A) deferoxamine (100 μg/ml) for 24 h. Bar, 50 μm.

h. Then, the medium was changed to fresh MEM and culture was continued for the indicated periods. PA production rates of cells treated with dithizone were constant throughout the experimental period, whereas that induced by deferoxamine began to disappear at 12 h and reached control level after 24 h cultivation in MEM. Therefore, in view of the reversibility, the mechanism of F9 differentiation by deferoxamine may be different from those of other chelators.

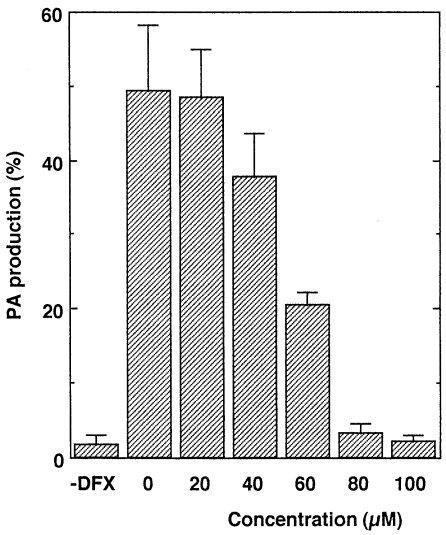

Fig. 3. Inhibition by Fe^{3+} of deferoxamine-induced PA production in F9 cells. The indicated concentrations of Fe^{3+} were preincubated with 50 μg/ml (76 μM) deferoxamine for 30 min at 37°C in the culture medium. F9 colonies were then exposed to the medium for 24 h and their PA production rates were assayed. −DFX indicates control culture without deferoxamine and Fe^{3+}. Values are means ± S.D. of triplicate cultures.

3.5. Cell proliferation and DNA fragmentation

As shown in Fig. 2, the number of cells was decreased in colonies treated with deferoxamine. To confirm this quantitatively, the number of cells was

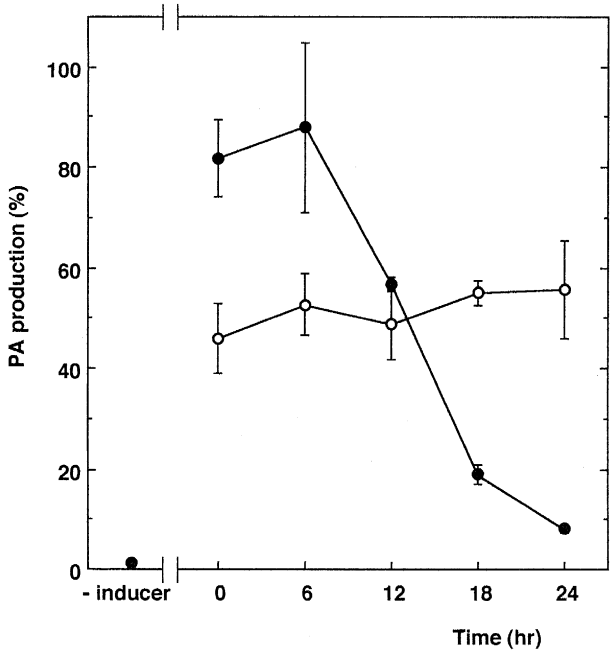

Fig. 4. Reversibility of differentiation induced by deferoxamine. F9 colonies were treated with 100 μg/ml deferoxamine (●) or 10 μg/ml dithizone (○) for 24 h. After washing, the cells were incubated without chelators for the indicated times. Then, PA production rate was assessed by the agar-overlay method. −inducer indicates control culture without chelators. All values represent means ± S.D. of triplicate cultures.

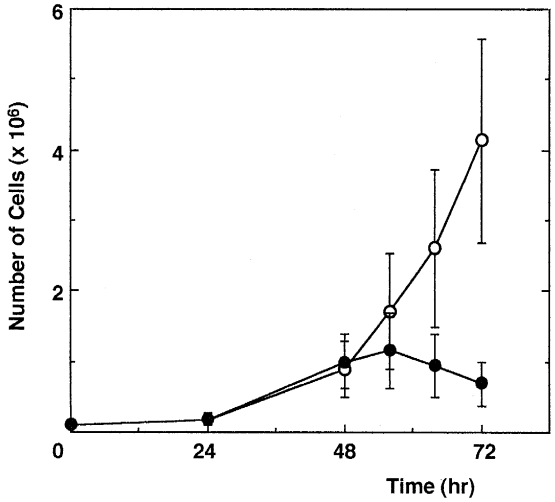

Fig. 5. Effects of deferoxamine on cell proliferation. F9 cells (1×10^5) were seeded into 6 cm dishes and cultured for 48 h. Then, culture medium was changed to fresh medium with (●) or without (○) 100 μg/ml deferoxamine. The number of cells was counted at the indicated times using a hematocytometer. Values are means ± S.D. of triplicate assays.

compared following cultivation in the presence or absence of deferoxamine. When F9 cells proliferated exponentially at 48 h after seeding, the medium was changed to fresh medium containing deferoxamine. Cells cultured without deferoxamine continued to grow over the experimental period, whereas deferoxamine inhibited the growth at 8 h after treatment and markedly reduced the cell number after 24 h (Fig. 5).

Further analysis revealed that the reduction of cell number was in part associated with the occurrence of apoptotic cell death. Total DNAs obtained from control and deferoxamine-treated F9 cells were electrophoresed in 2% agarose gels (Fig. 6). DNA ladder formation was observed in samples from deferoxamine-treated cells, with fragmented DNA in multiples of 170–180 base pairs which is typical of apoptosis. No signs of DNA fragmentation were observed in samples from control cells.

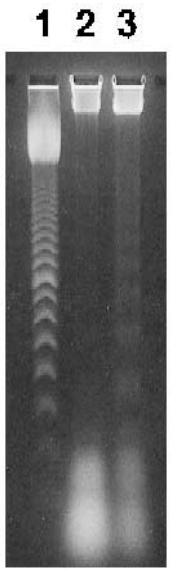

Fig. 6. Detection of DNA fragmentation in deferoxamine-treated F9 cells. Aliquots of 70 μg of DNA from deferoxamine (100 μg/ml)-treated (lane 3) and non-treated (lane 2) cells were electrophoresed in 2% agarose gels, and stained with ethidium bromide. Lane 1 is 100 bp ladder marker.

4. Discussion

Deferoxamine has been widely used for the treatment of various diseases such as thalassemia, iron overload, rheumatoid arthritis, and malaria [15–18]. It was reported that deferoxamine induced the differentiation of human leukemic cell line HL-60 to monocyte-macrophage cells [28,29]. However, other groups reported that deferoxamine did not induce differentiation of HL-60 cells [30,31]. Thus, the differentiation-inducing activity of deferoxamine in HL-60 cells is controversial. However, in this study, we observed differentiation-inducing activity of deferoxamine in F9 cells. These cells underwent a morphological change to the typically differentiated polygonal shape following treatment with deferoxamine (Fig. 2), which was quite similar to that induced by hinokitiol. Moreover, the differentiation-inducing activity of deferoxamine was dose- and time-dependent similarly to that of hinokitiol, and this agent acted more rapidly than retinoic acid again similarly to other chelators.

Previously, we reported that differentiation-inducing activity of chelators was specifically inhibited by preincubation with Fe^{3+} ions before addition to cell cultures [6]. The activity of deferoxamine was also dose-dependently inhibited by preincubation with Fe^{3+} (Fig. 3). It has been reported that deferoxamine binds equimolar amounts of Fe^{3+} [14]. Thus, deferoxamine may stoichiometrically chelate Fe^{3+} to form an inactive complex, and the remaining deferoxamine

functions to induce the differentiation of F9 cells. We speculate that the chelating ability of this agent causes the removal of extracellular and/or intracellular functional Fe^{3+}, resulting in F9 cell differentiation. In fact, it has been reported that deferoxamine reduces the intracellular iron level of some cell lines [29,32]. Iron ions in biological systems are always bound to proteinaceous ligands and are never present in free form [33]. About 60 and 30% of total iron ions bind to hemoglobin and ferritin, respectively [15]. There are many other proteins which contain iron ions (20 protein species or more) such as ribonucleotide reductase, transferrin and cytochromes [15,33]. We speculate that the main target of deferoxamine may be ribonucleotide reductase in F9 cells similarly to HL-60 cells [28,34] although we have no direct evidence to support this hypothesis, because it was reported that some chelators including deferoxamine inhibited the activity of this enzyme in many cell lines through the removal of iron ion from its active center [35–38]. Furthermore, our speculation seems reasonable due to both the participation of ribonucleotide reductase in DNA synthesis [39,40] and the inhibition of DNA synthesis in F9 cells induced to differentiate by various chelators [5].

The similarity of F9 cells differentiated by treatment with deferoxamine and other chelators suggested that the differentiation-inducing mechanisms of these agents may be the same. However, only those cells differentiated by deferoxamine showed a reversible change after its removal from the culture medium. The only other compound capable of inducing reversible differentiation of F9 cells reported to date is sodium butyrate [25]. The duration required for dedifferentiation was longer in cells differentiated by deferoxamine than by sodium butyrate. This reversibility may have originated from the characteristics of deferoxamine which acts as a reversible S-phase inhibitor [19]. Since deferoxamine is a hydrophilic chelator, it is also possible that the kinetics of deferoxamine in culture are different from those of other hydrophobic chelators.

It has also been reported that deferoxamine has cytotoxic and antiproliferative effects on many cell lines [20,41–43]. In this study, deferoxamine inhibited the proliferation of F9 cells (Fig. 5). Some investigators reported that deferoxamine induced apoptosis in HL-60 and K562 cells [28,44], and Haq et al. reported that the iron depletion caused apoptosis in CCRF-CEM cells [45]. The apoptosis induced by deferoxamine in F9 cells is thought to be the result of iron starvation by chelation. However, the relationships between differentiation and apoptosis remains to be clarified. In addition, it has been reported that iron ions significantly participate in radical production [46,47] and that there are iron-responsive elements in some mRNA sequences [48–50]. Therefore, it is likely that iron chelators added to cultures alter the levels of radicals and/or the regulation of gene expression in cells, resulting in cell differentiation and apoptosis. The mechanisms of induction of differentiation and apoptosis in F9 cells should be investigated in more detail to gain further insight into normal development and to develop novel agents for cancer chemotherapy.

References

[1] Bernstine, E.G., Hooper, M.L., Grandchamp, S. and Ephrussi, B. (1973) Proc. Natl. Acad Sci. USA 70, 3899–3903.
[2] Strickland, S. and Mahdavi, V. (1978) Cell 15, 393–403.
[3] Nishimune, Y., Kume, A., Ogiso, Y. and Matsushiro, A. (1983) Exp. Cell Res. 146, 439–444.
[4] Kondo, K., Watanabe, T., Sasaki, H., Uehara, Y. and Oishi, M. (1989) J. Cell Biol. 109, 285–293.
[5] Muto, N., Dota, A., Tanaka, T., Itoh, N., Okabe, M., Inada, A., Nakanishi, T. and Tanaka, K. (1995) Biol. Pharm. Bull. 18, 1576–1579.
[6] Tanaka, T., Muto, N., Itoh, N., Dota, A., Nishina, Y., Inada, A. and Tanaka, K. (1995) Res. Comm. Mol. Pathol. Pharmacol. 90, 211–220.
[7] Strickland, S., Smith, K.K. and Marotti, K.R. (1980) Cell 21, 347–355.
[8] Solter, D. and Knowles, B.B. (1978) Proc. Natl. Acad. Sci. USA 75, 5565–5569.
[9] Sleigh, M.J. (1989) Biochem. Soc. Symp. 55, 1–12.
[10] Iwai, A.S., Kosaka, M., Nishina, Y., Sumi, T., Sakuda, M. and Nishimune, Y. (1993) Exp. Cell Res. 205, 39–45.
[11] Pikarsky, E., Sharir, H., Ben-Shushan, E. and Bergman, Y. (1994) Mol. Cell. Biol. 140, 1026–1038.
[12] Hogan, B.L.M., Barlow, D.P. and Tilly, R. (1983) Cancer Surveys 2, 116–140.
[13] Koberle, H. (1964) Ann. NY Acad. Sci. 119, 758–768.
[14] Goodwin, J.F. and Whitten, C.F. (1965) Nature 205, 281–283.
[15] Kontoghiorghes, G.J. (1995) Toxicol. Lett. 80, 1–18.
[16] Kontoghiorghes, G.J. (1985) Lancet i, 817.

[17] Jackson, T.W., Ling, L.J. and Washington, V. (1995) J. Toxicol.-Clin. Toxicol. 33, 325–329.
[18] Muntane, J., Puig-Parellada, P. and Mitjavia, M.T. (1995) J. Lab. Clin. Med. 126, 435–443.
[19] Lederman, H.M., Cohen, A., Lee, J.W.W., Freeman, M.H. and Gelfand, E.W. (1984) Blood 64, 748–753.
[20] Weinberg, K. (1990) Am.J. Pediatric Hematol. Oncol. 12, 9–13.
[21] Fukuchi, K., Tomoyasu, S., Watanabe, K., Watanabe, H., Takagi, Y., Tsuruoka, N. and Gomi, K. (1993) Biochem. Mol. Biol. Int. 30, 403–409.
[22] Donfrancesco, A., Deb, G., De Sio, L., Cozza, R., and Castellano, A. (1993) Acta Haematol. 95, 66–69.
[23] Hoyes, K.P., Hider, R.C. and Porter, J.B. (1992) Cancer Res. 52, 4591–4599.
[24] Jones, P., Benedict, W., Strickland, S. and Reich, E. (1975) Cell 5, 325–329.
[25] Kosaka, M., Nishina, Y., Takeda, M., Matsumoto, K. and Nishimune, Y. (1991) Exp. Cell Res. 192, 46–51.
[26] Ramachandra, S. and Studzinski, G.P. (1995) in Cell Growth and Apoptosis (Studzinski, G.P., ed.), pp. 119–142, IRL Press, Oxford.
[27] McCue, P.A., Gubler, M.L., Sherman, M.I. and Cohen, B.N. (1984) J. Cell Biol. 98, 602–608.
[28] Kaplinsky, C., Estrov, Z., Freedman, M.H., Gelfand, E.W. and Cohen, A. (1987) Leukemia 1, 437–441.
[29] Seligman, P.A., Kovar, J. and Gelfand, E.W. (1992) Pathobiology 60, 19–26.
[30] Tomoyasu, S., Nakamaki, T., Suzuki, K. and Tsuruoka, N. (1987) Acta Haematol. JPN 50, 568–574.
[31] Voest, E.E., Rooth, H., Neijt, J.P., van Asbeck, B.S. and Marx, J.J.M. (1993) Cell Prolif. 26, 77–88.
[32] Rothman, R.J., Serroni, A. and Farber, J.L. (1992) Mol. Pharmacol. 42, 703–710.
[33] Kontoghiorghes, G.J. and Weinberg, E.D. (1995) Blood Rev. 9, 33–45.
[34] Barankiewicz, J. and Cohen, A. (1987) Biochem. Pharmacol. 36, 2343–2347.
[35] Kontoghiorghes, G.J., Piga, A. and Hoffbrand, A.V. (1986) Hematological Oncol. 4, 195–204.
[36] Oblender, M. and Carpentieri, U. (1991) J. Cancer Res. Clin. Oncol. 117, 444–448.
[37] Fontecave, M., Nordlund, P., Eklund, H. and Reichard, P. (1992) Adv. Enzymol. 65, 148–183.
[38] Nocentini, G., Federici, F., Franchetti, P. and Barzi, A. (1993) Cancer Res. 53, 19–26.
[39] Holmgren, A. (1979) J. Biol. Chem. 254, 3664–3671.
[40] Reichard, P. (1993) Science 260, 1773–1777.
[41] Nyholm, S., Mann, G.J., Johansson, G., Bergeron, R.J.,Grasloud, A. and Thelander, L. (1993) J. Biol. Chem. 268, 26200–26205.
[42] Tomoyasu, S., Fukuchi, K., Yajima, K., Watanabe, K., Suzuki, H., Kawakami, K., Gomi, K. and Tsuruoka, N. (1993) Anticancer Res. 13, 407–410.
[43] Lee, Y.S. and Werster, R.D. (1995) Toxicol. Lett. 78, 67–71.
[44] Hileti, D., Panayiotidis, P. and Hoffbrand, A.V. (1995) Br. J. Haematol. 89, 181–187.
[45] Haq, R.U., Wereley, J.P. and Chitambar, C.R. (1995) Exp. Hematol. 23, 428–432.
[46] McCord, J.M. and Day, E.D. (1978) FEBS Lett. 86, 139–142.
[47] Halliwell, B. and Gutteridge, J.M.C. (1984) Biochem. J. 219, 1–14.
[48] Klausner, D., Rouault, T.A. and Harford. J.B. (1993) Cell 72, 19–28.
[49] Basilion, J.P., Rouault, T.A., Massinople, C.M., Klausner, R.D. and Burgess, W.H. (1994) Proc. Natl. Acad. Sci. USA 91, 574–578.
[50] Ferreira, G.C. (1995) Biochem. Biophys. Res. Comm. 214, 875–878.