

Human cytochrome *c* enters murine J774 cells and causes G₁ and G₂/M cell cycle arrest and induction of apoptosis

Yoshinori Hiraoka^a, Ana Teresa Granja^{a,c}, Arsenio M. Fialho^{a,b,c},
Beatrix G. Schlarb-Ridley^{a,b}, Tapas K. Das Gupta^b,
Ananda M. Chakrabarty^a, Tohru Yamada^{a,b,*}

^a Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL, USA

^b Department of Surgical Oncology, University of Illinois College of Medicine, Chicago, IL, USA

^c Biological Sciences Research Group, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal

Received 11 October 2005

Available online 24 October 2005

Abstract

Cytochrome *c* is well known as a carrier of electrons during respiration. Current evidence indicates that cytochrome *c* also functions as a major component of apoptosomes to induce apoptosis in eukaryotic cells as well as an antioxidant. More recently, a prokaryotic cytochrome *c*, cytochrome *c*₅₅₁ from *Pseudomonas aeruginosa*, has been shown to enter in mammalian cells such as the murine macrophage-like J774 cells and causes inhibition of cell cycle progression. Much less is known about such functions by mammalian cytochromes *c*, particularly the human cytochrome *c*. We now report that similar to *P. aeruginosa* cytochrome *c*₅₅₁, the purified human cytochrome *c* protein can enter J774 cells and induce cell cycle arrest at the G₁ to S phase, as well as at the G₂/M phase at higher concentrations. Unlike *P. aeruginosa* cytochrome *c*₅₅₁ which had no effect on the induction of apoptosis, human cytochrome *c* induces significant apoptosis and cell death in J774 cells, presumably through inhibition of the cell cycle at the G₂/M phase. When incubated with human breast cancer MCF-7 and normal mammary epithelial cell line MCF-10A1 cells, human cytochrome *c* entered in both types of cells but induced cell death only in the normal MCF-10A1 cells. The ability of human cytochrome *c* to enter J774 cells was greatly reduced at 4 °C, suggesting energy requirement in the entry process.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Cell cycle arrest; Human cancer; Apoptosis; Protein entry; Cupredoxins; Cytochromes; p16^{Ink4a} tumor suppressor

The roles of cytochrome *c* in respiration and various oxidative processes, including its role in oxidative stress and as an antioxidant, are well known [1–3]. Also well known is the function of cytochrome *c* as an inducer of apoptosis in eukaryotic cells when released from the mitochondria to the cell cytosol [4,5]. The multifunctional nature and the presence of separate domains for various activities in cytochrome *c* are quite clear from the fact that the apoptotic effects can be separated from the other functions involving respiration or antioxidant activities [6–8]. Recently we reported that a prokaryotic cytochrome *c*,

cytochrome *c*₅₅₁ from *Pseudomonas aeruginosa*, could enter mammalian cells such as the murine reticulum cell sarcoma J774 cells with macrophage like properties [9] and cause G₁ to S phase cell cycle arrest without inducing apoptosis [10]. Interestingly, eukaryotic cytochromes such as horse and bovine cytochrome *c* were also shown to induce apoptosis but not inhibition of cell cycle progression in J774 cells [10]. Since human cytochrome *c* has recently been purified and the recombinant protein has been shown to activate caspase-3 in a cell-free caspase activation assay [11], it was of interest to us to examine if the human cytochrome *c* can enter mammalian cells, including normal human cells, to exert cytotoxicity. Here, we report the ability of human cytochrome *c* to enter both the murine J774 cells and the human mammary epithelial MCF-10A1 cells. While

* Corresponding author. Fax: +1 312 996 6415.

E-mail address: tohru@uic.edu (T. Yamada).

human cytochrome *c* could strongly inhibit cell cycle progression resulting in apoptosis of the J774 cells, it had very low cell cycle inhibitory activity in the human epithelial cells, although it demonstrated significant cytotoxicity.

Materials and methods

Purification of human cytochrome *c* and TUNEL assay. Human cytochrome *c* was purified as described by Olteanu et al. [11]. The effect of human cytochrome *c* on cell cycle progression in murine J774 cells was determined by flow cytometry as detailed earlier using propidium iodide staining [10]. For determination of apoptotic cell death, J774 cells were treated with various concentrations of human cytochrome *c* for different time periods, washed with phosphate-buffered saline (PBS), and fixed with 1% paraformaldehyde. The cells were stained by using an APO-DIRECT apoptosis kit (Phoenix Flow Systems, San Diego) and analyzed by flow cytometry as described earlier [10].

Cell culture. J774, MCF-10A1, and cancer cells MCF-7 or Mel-2 were cultured as described earlier [10,12].

Entry of human cytochrome *c* in J774 and human normal and cancer cells. For entry experiments, human cytochrome *c*, azurin or rusticyanin was labeled chemically with the red fluorescent dye Alexa Fluor 568 (Molecular Probes, San Diego) and incubated with J774 or human normal MCF-10A1 or breast cancer MCF-7 or melanoma Mel-2 cells for 1 h at 37 °C. The treated cells were fixed with methanol at –20 °C, stained with DAPI for nuclear staining, and confocal microscopic images were taken as described earlier [12]. For competition experiments, the Alexa Fluor 568-conjugated human cytochrome *c* at 200 µg/ml was incubated for 1 h at 37 °C with J774 cells pretreated with 200 or 800 µg/ml of other unlabeled cytochromes such as cytochrome *c*₅₅₁ or cytochrome *f* or a cupredoxin such as azurin at 37 °C for 1 h before the confocal microscopic images were taken.

Western blot analysis. J774 cells were treated with 200 µg/ml of human cytochrome *c* for various time periods at 37 °C after which cell lysates were prepared as described previously [10]. Thirty micrograms of protein from each lysate was separated by SDS-PAGE, transferred to poly(vinylidene difluoride) membranes, and probed with primary antibodies against cyclins, CDKs, and tumor suppressor proteins. After mixing with appropriate peroxidase-conjugated secondary antibody, ECL detection system (GE Healthcare) was used [10].

MTT assay. For measurement of the cytotoxic activity of human cytochrome *c* in normal MCF-10A1 cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was conducted as described previously [13].

Results and discussion

Cell cycle arrest and induction of apoptosis by human cytochrome *c*

Our recent demonstration that a prokaryotic cytochrome *c*, viz., cytochrome *c*₅₅₁ from *P. aeruginosa*, enters J774 cells and causes inhibition of cell cycle progression at the G₁ to S phase [10], raised the question if vertebrate cytochrome *c* can act in a similar manner. In the same report [10], we demonstrated that vertebrate cytochrome *c* such as bovine or horse cytochrome *c* can enter J774 cells and induce apoptosis, but not cell cycle arrest. In contrast, another bacterial redox protein, a cupredoxin rusticyanin, was shown to enter J774 cells causing G₁ arrest by depleting CDK2 but not CDK4 or CDK6 [13]. Rusticyanin, however, had no significant effect on the induction of apoptosis in J774 cells [13]. The ability of a bacterial redox protein to induce either cell cycle arrest or apoptosis was shown to depend on relative cell surface hydrophobicity of the protein. For example, another bacterial cupredoxin, azurin, was shown to induce apoptosis in J774 cells but little cell cycle arrest [14]. In contrast, a mutant form of azurin, the M44KM64E azurin, where two hydrophobic methionine residues on the surface were replaced by two charged amino acids, elicited little induction of apoptosis in J774 cells but triggered inhibition of cell cycle progression [15]. It was therefore of interest to us to examine how human cytochrome *c* would act

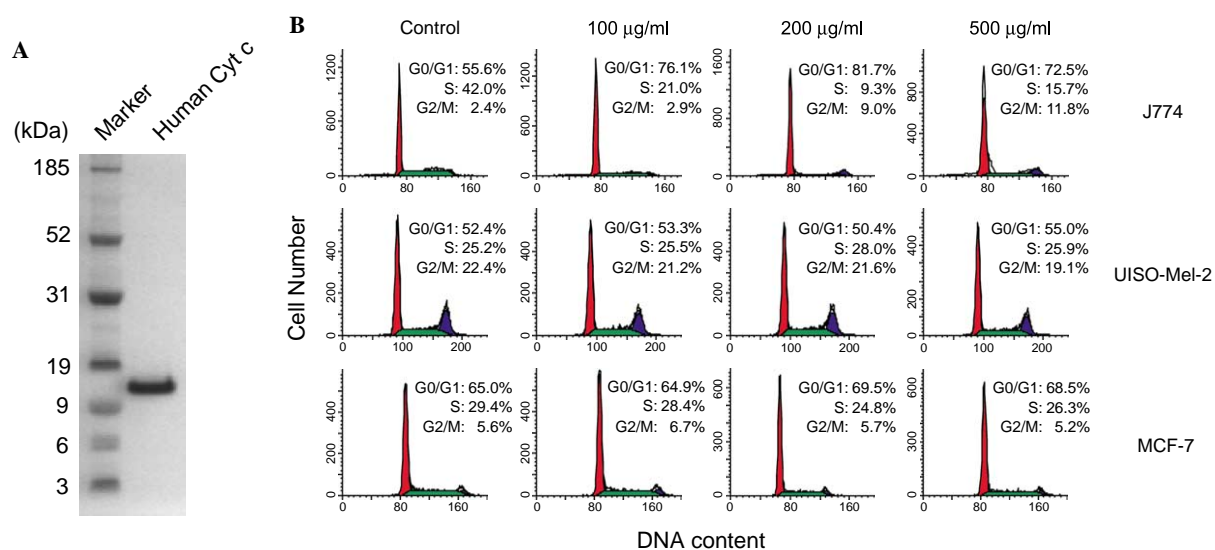


Fig. 1. (A) Isolation of human cytochrome *c*. Purified heme-containing human cytochrome *c* was applied on SDS-PAGE and visualized by Coomassie blue staining. (B) Effect of human cytochrome *c* on cell cycle progression in J774, UIISO-Mel-2, and MCF-7 cells. The cells were harvested either without treatment (control) or on treatment with different concentrations of human cytochrome *c* (100, 200, and 500 µg/ml). After DNA staining with propidium iodide (50 µg/ml), cells were analysed by flow cytometry [10]. Percentages of cells at G₀/G₁, S, and G₂/M phases were determined by using MODFIT software.

towards J774 or cells of a normal human mammary epithelial cell line MCF-10A1 [16]. We used a construct that was previously used by Pielak and co-workers for expression of the human cytochrome *c* gene [11] which produced a protein with a high degree of purity and homogeneous mobility on SDS-PAGE (Fig. 1A). We incubated this purified protein at various concentrations (100, 200, and 500 $\mu\text{g/ml}$) with J774 and two human cancer cells UIISO-Mel-2 (melanoma) and MCF-7 (breast cancer). Similar to the activity of cytochrome *c*₅₅₁, human cytochrome *c* inhibited cell cycle in J774 cells at the G₁ to S phase, but only to a limited degree. Unlike cytochrome *c*₅₅₁, human cytochrome *c* also had an inhibitory effect at the G₂/M phase at higher concentrations (200 $\mu\text{g/ml}$ and higher). No such effect was seen with the human cancer cells, either at the G₀/G₁ phase or at the G₂/M phase (Fig. 1B). Cancer cells such as MCF-7 are known to

override inhibitory effects at the G₁ to S phase since they modulate the retinoblastoma function in such a way that the E2F activity is no longer susceptible to inhibitory effects at the G₁ to S phase [17,18].

The *P. aeruginosa* cytochrome *c*₅₅₁ had no significant effect on the G₂/M phase and very little apoptotic activity towards J774 cells [10]. Given the inhibitory effect of human cytochrome *c* on the G₂/M phase at concentrations of 200 $\mu\text{g/ml}$, and since inhibition of G₂/M phase often leads to induction of apoptosis [19,20], we checked the ability of human cytochrome *c* to induce apoptosis in J774 cells as well as the human cancer cells UIISO-Mel-2 and MCF-7. We used TUNEL assay using flow cytometry (Fig. 2A) to measure apoptosis. While human cytochrome *c* had very little effect on the induction of apoptosis in human cancer cells (Figs. 2A and B), it had significant apoptotic effect on J774 cells at 200 $\mu\text{g/ml}$ and higher concentrations (Figs.

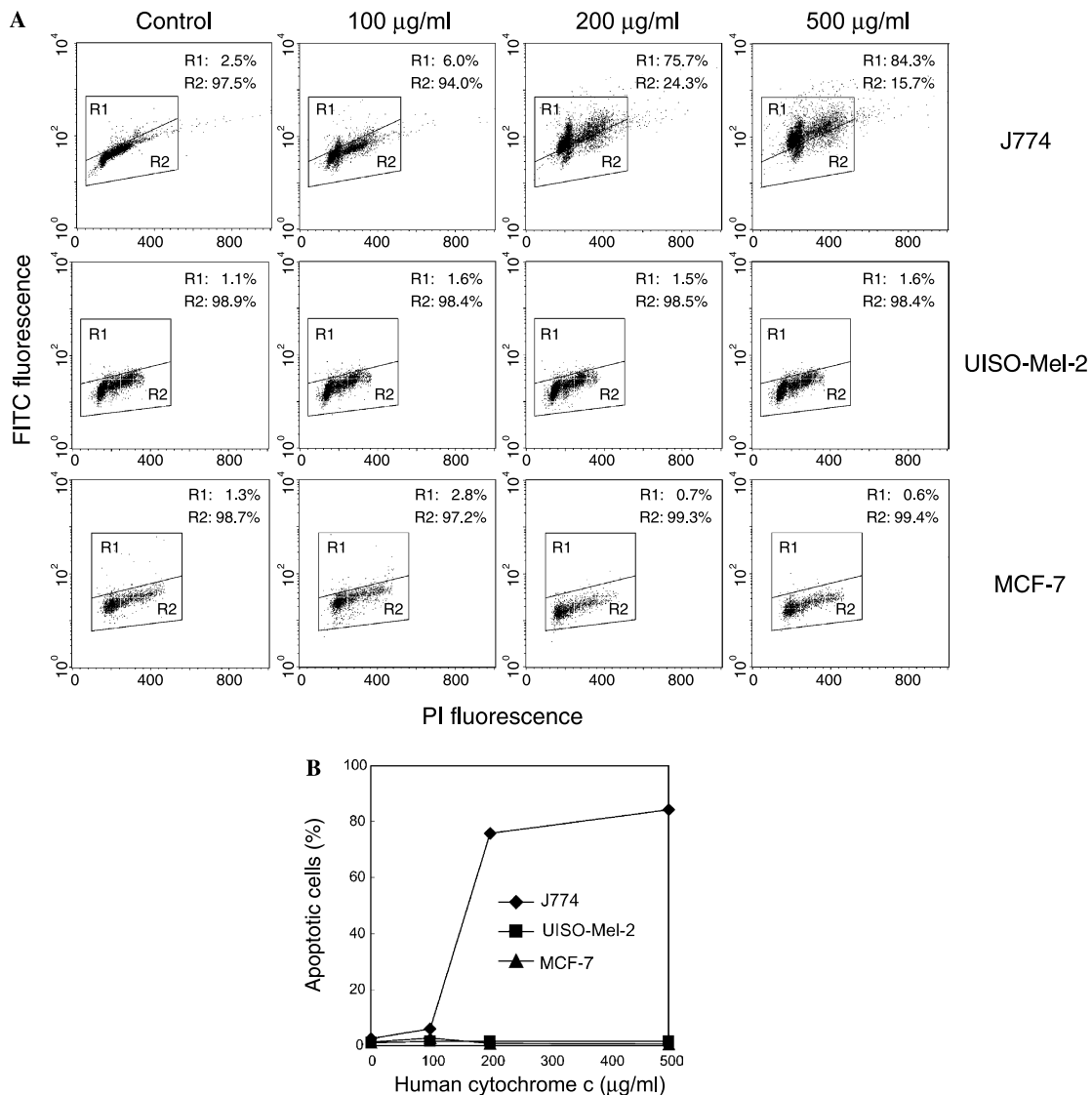


Fig. 2. (A) Induction of apoptosis by human cytochrome *c* towards J774, UIISO-Mel-2, and MCF-7 cells. The cells were untreated (control) or treated with human cytochrome *c* (100–500 $\mu\text{g/ml}$ for 24 h) and analyzed by using APO-DIRECT apoptosis kit (Phoenix Flow Systems). R1, apoptotic cells; R2, nonapoptotic cells. (B) The results of (A) are plotted graphically to demonstrate apoptotic activity of the human cytochrome *c*.

2A and B), where the inhibition at the G₂/M phase of the cell cycle was the strongest.

Human cytochrome c can enter J774 cells and requires energy for such entry

The fact that purified human cytochrome *c* could induce cell cycle arrest and apoptosis strongly suggested that either it bound on the surface of J774 cells with specific receptors to modulate a signaling pathway or enter the cytosol of the J774 cells to directly induce cell cycle arrest or apoptosis. It was also of interest to determine if the binding and/or entry of human cytochrome *c* is an energy-requiring process involving receptor binding. We therefore conjugated human cytochrome *c* with red fluorescing Alexa Fluor 568 dye and incubated with J774 cells at 100 and 200 µg/ml concentrations at 37 °C for 1 h.

The human cytochrome *c* had limited entry at 100 µg/ml but was clearly visible in the cytosol at 200 µg/ml. The nucleus was stained blue with DAPI (Fig. 3A). The entry was severely inhibited when the incubation temperature was 4 °C, suggesting that the entry process required energy (Fig. 3A). To determine if human cytochrome *c* entry in

J774 cells was mediated by receptor binding, we preincubated the cells with unlabeled human cytochrome *c* at 200 and 800 µg/ml for 1 h at 37 °C. We then added 200 µg/ml Alexa Fluor 568-labeled human cytochrome *c* and incubated for a further 1 h period at 37 °C. The preincubation with unlabeled human cytochrome *c*, particularly at 800 µg/ml concentration, severely and competitively inhibited the entry of the labeled human cytochrome *c*, suggesting saturation of putative receptor binding sites for human cytochrome *c*.

To determine if other cytochromes or even a cupredoxin such as azurin, which is known to enter mammalian cells including J774 cells [12], could inhibit human cytochrome *c* entry, the competitive entry of Alexa Fluor 568-labeled 200 µg/ml human cytochrome *c* at 37 °C for 1 h was evaluated in J774 cells preincubated with 200 or 800 µg/ml of unlabeled redox proteins. For this purpose, the proteins used were cytochrome *c*₅₅₁, cytochrome *f*, a photosynthetic redox partner of the cupredoxin plastocyanin from the thermophilic cyanobacterium *Phormidium laminosum* where during photosynthesis the electrons are transferred from cytochrome *f* of the cytochrome *bf* complex via plastocyanin to photosystem I [21], or azurin. While the

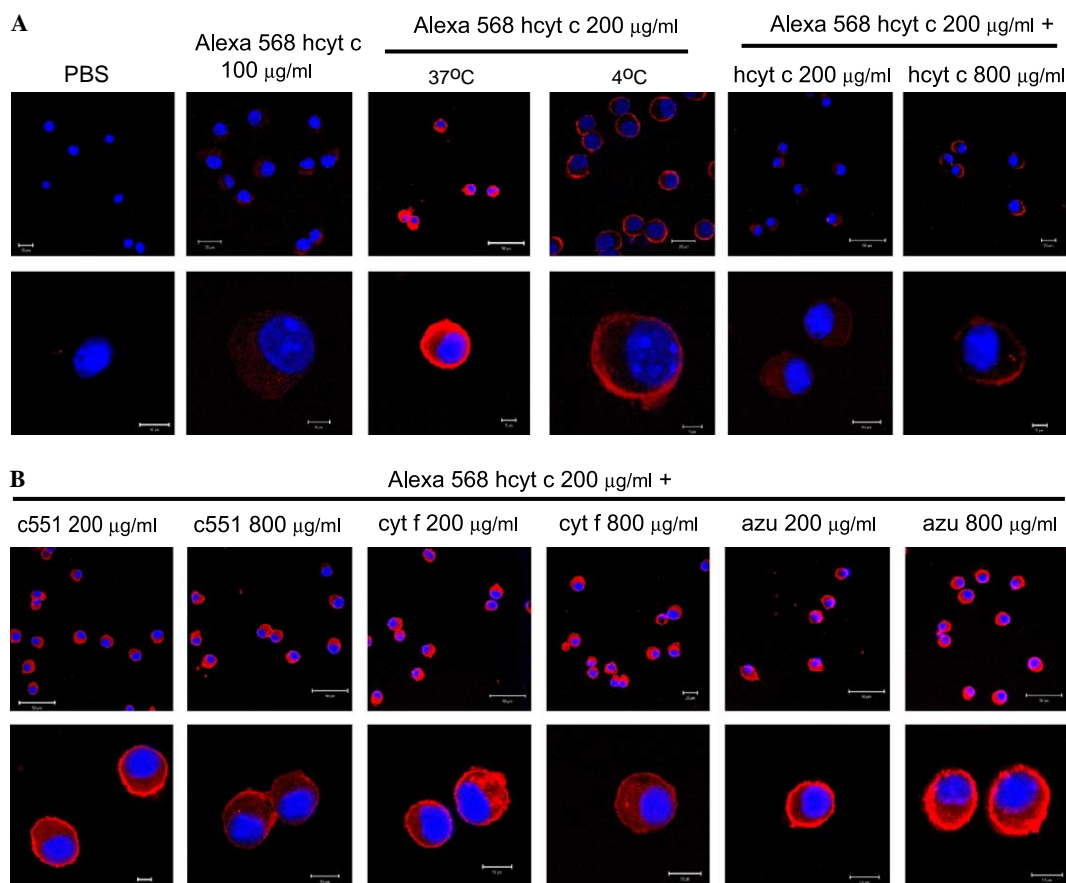


Fig. 3. (A) Entry of Alexa Fluor 568-conjugated human cytochrome *c*. J774 cells were treated with 200 µg/ml of Alexa Fluor 568-labeled human cytochrome *c* for 1 h at 37 °C or at 4 °C. After washing with PBS twice, cells were fixed and observed by confocal microscopy as described earlier [12]. (B) J774 cells were pretreated with 200 and 800 µg/ml of unlabeled proteins such as human cytochrome *c*, cytochrome *c*₅₅₁, cytochrome *f*, and azurin for 1 h at 37 °C. The cells were then washed with PBS and incubated with 200 µg/ml of Alexa Fluor 568-conjugated human cytochrome *c* before being checked by confocal microscopy as shown in (A).

two cytochromes had a low inhibitory effect at high concentrations (800 $\mu\text{g/ml}$), azurin had very little effect (Fig. 3B), suggesting that the entry of human cytochrome *c* was not significantly affected in the presence of high concentrations of other cytochromes or a cupredoxin such as azurin.

The ability of the human cytochrome *c* to enter in J774 cells to modulate both cell cycle (Fig. 1B) and the induction of apoptosis (Figs. 2A and B) without having any such

effects on the human melanoma UIISO-Mel-2 or the breast cancer MCF-7 (Figs. 1B and 2B) raised an interesting question: is such lack of effect in cancer cells due to a lack of entry of human cytochrome *c* in human cancer cells? What about any effect of purified human cytochrome *c* on normal human cells? We previously demonstrated the entry of the cupredoxins azurin and rusticyanin in the Mel-2 and MCF-7 cells, leading to cytotoxic effects [12,13]. We demonstrated that while rusticyanin could enter into both types of

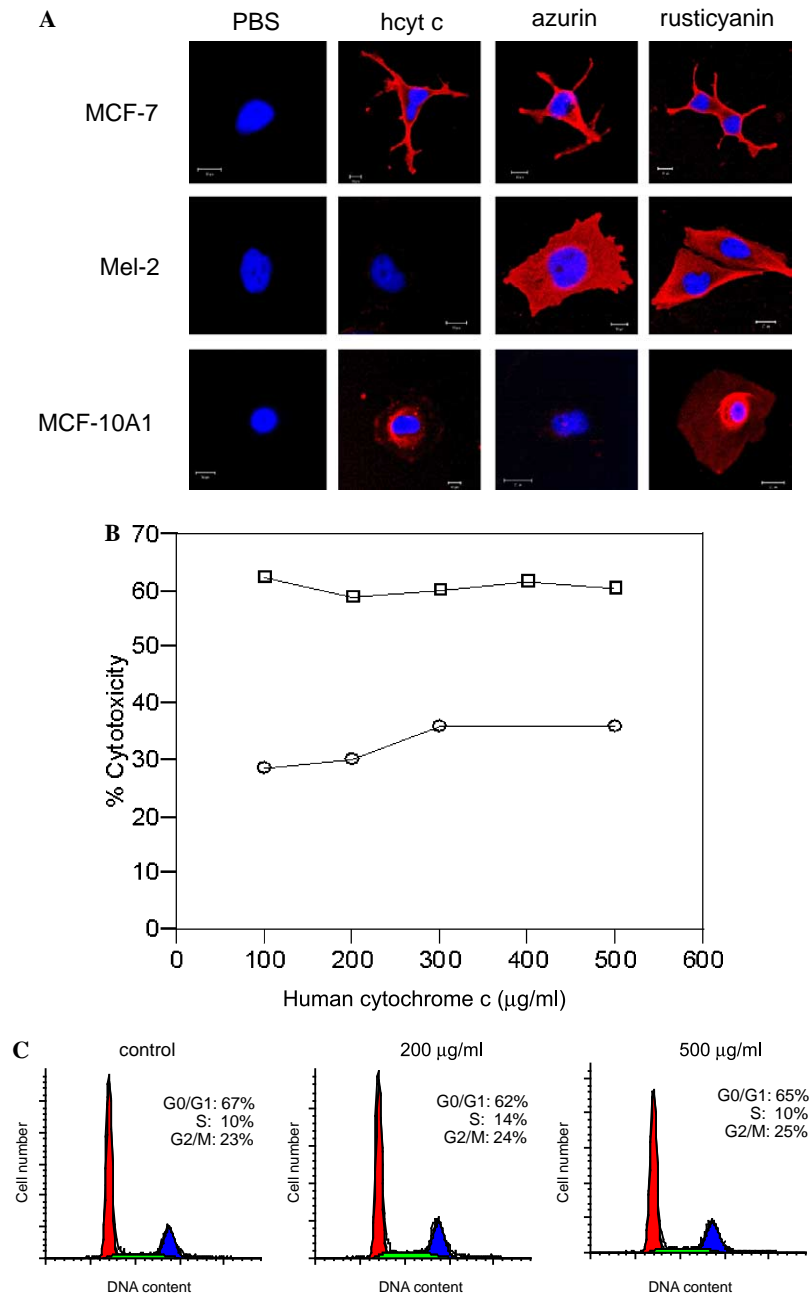


Fig. 4. (A) Mel-2, MCF-7, and MCF-10A1 cells were treated with 200 $\mu\text{g/ml}$ of several Alexa Fluor 568-conjugated proteins such as azurin, rusticyanin, and human cytochrome *c* at 37 $^{\circ}\text{C}$ for 1 h. Entry of such proteins was checked by confocal microscopy. (B) Cytotoxic effects of human cytochrome *c* on MCF-10A1 cells. Cells were treated with various concentrations of human cytochrome *c* for 24 h (circle) and 48 h (square) prior to the determination of cytotoxicity by MTT assay. (C) Effect of human cytochrome *c* on cell cycle progression in MCF-10A1 cells. The cells were harvested either without treatment (control) or after treatment with human cytochrome *c* at 200 or 500 $\mu\text{g/ml}$. Percentages of MCF-10A1 cells at G₀/G₁, S, and G₂/M phases were determined after 48 h incubation.

cancer cells and the immortalized normal mammary epithelial cells MCF-10A1, azurin was proficient in entering the human cancer cells but deficient in entering the normal cells such as MCF-10A1 [12]. We therefore made a comparative study of the entry of human cytochrome *c*, azurin, and rusticyanin in MCF-7, Mel-2, and MCF-10A1 cells. As shown earlier [12], rusticyanin entered in all the three types of cells, although most of it was found to be sequestered surrounding the nucleus in the normal MCF-10A1 cells. Azurin entered both MCF-7 and Mel-2 cells efficiently and was localized in the cytosol. As demonstrated earlier [12], azurin was very inefficient in entering the normal MCF-10A1 cells (Fig. 4A). Human cytochrome *c* was found to enter MCF-7 cells and localized in the cytosol. Interestingly, however, it was very inefficient in entering the Mel-2 cells (Fig. 4A). It is not known if the inability of human cytochrome *c* to enter Mel-2 cells is the reason for its lack of induction of cytotoxic effects in such cells. Human cytochrome *c* could enter the normal mammary MCF-10A1 cells, but like rusticyanin, was sequestered in the perinuclear space (Fig. 4A).

Does entry of human cytochrome *c* in normal MCF-10A1 type of cells have any physiological consequences? Incubation of MCF-10A1 cells for 24 h with various concentrations of purified human cytochrome *c* demonstrated significant cytotoxicity even at 100 µg/ml (Fig. 4B). Such cytotoxicity increased further on prolonged incubation (48 h). No effect of human cytochrome *c* was, however, discernible on cell cycle progression in MCF-10A1 cells, even at 500 µg/ml concentration (Fig. 4C).

The cell cycle effect of human cytochrome c in J774 cells is modulated by p16^{Ink4a} levels

We previously reported [10] that the prokaryotic cytochrome *c*₅₅₁ from *P. aeruginosa* exerted its effect on J774 cells through enhancement of the levels of p16^{Ink4a}, a known inhibitor of cell cycle at the G₁ to S phase [22]. We therefore determined the intracellular levels of various cyclins, CDKs, and tumor suppressors as described earlier for cytochrome *c*₅₅₁ [10]. When J774 cells were incubated with 200 µg/ml of human cytochrome *c* for various times

as indicated, lysates were prepared from the treated cells and the levels of various cyclins, CDKs, and tumor suppressors determined by Western blotting [10], the CDK4 and cyclin D1 levels were seen to be drastically reduced with increasing times of incubation (Fig. 5). While no significant effect was seen with other cyclins, CDKs, and tumor suppressor protein levels (data not shown), the p16^{Ink4a} levels were seen to go up with increasing periods of incubation, while the actin levels remained fairly constant (Fig. 5). Such data demonstrated that human cytochrome *c* activity on cell cycle progression in J774 cells mimics that of the prokaryotic cytochrome *c*₅₅₁, a major difference being the ability of human cytochrome *c* to cause significant inhibition at the G₂/M phase at higher concentrations, an effect not seen with cytochrome *c*₅₅₁ even at very high concentrations [10].

The ability of human cytochrome *c* to enter normal human cells such as MCF-10A1 to induce cytotoxicity (Figs. 4A and B) appears to suggest that human cytochrome *c* may play an additional role other than the induction of apoptosis when released from the mitochondria on exposure to death signals. In necrotizing cells, the release of significant quantities of cytochrome *c* to the outside fluid may allow its entry to the neighboring cells, leading to their cell death and perhaps limiting the spread of the necrotizing agent. The mode of entry of human cytochrome *c* and the nature of putative receptors for such entry, including why certain cancer cells such as Mel-2 do not allow human cytochrome *c* entry inside such cells, are open questions right now. It is clear, however, that redox proteins such as cupredoxins and cytochromes, apart from their roles as electron transfer agents, play important roles also in mammalian cell entry and modulation of cell cycle or cell death events.

Acknowledgments

We are indebted to and we thank Drs. Gary Pielak and Alina Olteanu of the University of North Carolina, Chapel Hill, for generously sharing with us their *E. coli* strain hyperexpressing the human cytochrome *c* gene. The investigation was supported by a grant from CDG Therapeutics as part of a sponsored research agreement with the University of Illinois at Chicago and in part by a Public Health Service Grant ES-04050-19 from the National Institute of Environmental Health Sciences. A.T.G. acknowledges a Ph.D. Grant (BD/10328/2002) from Fundacao Ciencia e Tecnologia, Portugal.

References

- [1] R.G. Moore, G.W. Pettigrew, *Cytochrome c: Evolutionary, Structural, and Physicochemical Aspects*, Springer-Verlag, Berlin, 1990.
- [2] M. Hashimoto, A. Takeda, J.L. Hsu, T. Takenouchi, E. Masliah, Role of cytochrome *c* as a stimulator of α -synuclein aggregation in Lewy Body disease, *J. Biol. Chem.* 274 (1999) 28849–28852.
- [3] S.S. Korshunov, B.F. Krasnikov, M.O. Pereverzev, V.P. Skulachev, The antioxidant functions of cytochrome *c*, *FEBS Lett.* 462 (1999) 192–198.

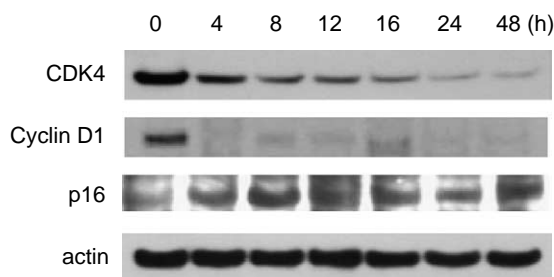


Fig. 5. Effect of human cytochrome *c* on cell cycle regulatory proteins in J774 cells. The J774 cells were treated with 200 µg/ml of human cytochrome *c* for the indicated times, whole cell lysates were prepared and analyzed by Western blotting for the indicated proteins as described earlier [10].

- [4] C. Adrian, S.J. Martin, The mitochondrial apoptosome: a killer unleashed by the cytochrome *c*, *Trends Biochem. Sci.* 26 (2001) 390–397.
- [5] G.V. Loo, X. Saelens, M.V. Gorp, M. MacFarlane, S.J. Martin, P. Vandenabeele, The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet, *Cell Death Differ.* 9 (2002) 1031–1042.
- [6] R.M. Kluck, S.J. Martin, B.M. Hoffman, J.S. Zhou, D.R. Green, D.D. Newmeyer, Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system, *EMBO J.* 16 (1997) 4639–4649.
- [7] R.M. Kluck, L.M. Ellerby, H.M. Ellerby, S. Naiem, M.P. Yaffe, E. Margoliash, D. Bredesen, A.G. Mauk, F. Sherman, D.D. Newmeyer, Determinants of cytochrome *c* proapoptotic activity, *J. Biol. Chem.* 275 (2000) 16127–16133.
- [8] Z.K. Abdullaev, M.E. Bodrova, B.V. Chernyak, D.A. Dolgikh, R.M. Kluck, M.O. Pereverzev, A.S. Arseniev, R.G. Efremov, M.P. Kirpichnikov, E.N. Mokhova, D.D. Newmeyer, H. Roder, V.P. Skulachev, A cytochrome *c* mutant with high electron transfer and antioxidant activities but devoid of apoptogenic effect, *Biochem. J.* 362 (2002) 749–754.
- [9] P. Ralph, I. Nakoinz, Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line, *Nature* 257 (1975) 393–394.
- [10] Y. Hiraoka, T. Yamada, M. Goto, T.K. Das Gupta, A.M. Chakrabarty, Modulation of mammalian cell growth and death by prokaryotic and eukaryotic cytochrome *c*, *Proc. Natl. Acad. Sci. USA* 101 (2004) 6427–6432.
- [11] A. Olteanu, C.N. Patel, M.M. Dedmon, S. Kennedy, M.W. Linhoff, C.M. Minder, P.R. Potts, M. Deshmukh, G.J. Pielak, Stability and apoptotic activity of recombinant human cytochrome *c*, *Biochem. Biophys. Res. Commun.* 312 (2003) 733–740.
- [12] T. Yamada, A.M. Fialho, V. Punj, L. Bratescu, T.K. Das Gupta, A.M. Chakrabarty, Internalization of bacterial redox protein azurin in mammalian cells: entry domain and specificity, *Cell Microbiol.* 7 (2005) 1418–1431.
- [13] T. Yamada, Y. Hiraoka, T.K. Das Gupta, A.M. Chakrabarty, Rusticyanin, a bacterial electron transfer protein, causes G1 arrest in J774 and apoptosis in human cancer cells, *Cell Cycle* 3 (2004) 1182–1187.
- [14] T. Yamada, M. Goto, V. Punj, O. Zaborina, K. Kimbara, T.K. Das Gupta, A.M. Chakrabarty, The bacterial redox protein azurin induces apoptosis in J774 macrophages through complex formation and stabilization of the tumor suppressor protein p53, *Infect. Immunol.* 70 (2002) 7054–7062.
- [15] T. Yamada, Y. Hiraoka, M. Ikehata, K. Kimbara, B.S. Avner, T.K. Das Gupta, A.M. Chakrabarty, Apoptosis or growth arrest: modulation of tumor suppressor p53's specificity by bacterial redox protein azurin, *Proc. Natl. Acad. Sci. USA* 101 (2004) 4770–4775.
- [16] H.D. Soule, T.M. Maloney, S.R. Wolman, W.D. Peterson, R. Brenz, C.M. McGrath, J. Russo, R.J. Pauley, R.F. Jones, S.C. Brooks, Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10, *Cancer Res.* 50 (1990) 6075–6086.
- [17] M.S. Orr, N.C. Watson, S. Sundaram, J.K. Randolph, P.T. Jain, D.A. Gewirtz, Ionizing radiation and teniposide increase p21^{waf1/cip1} and promote Rb dephosphorylation but fail to suppress E2F activity in MCF-7 breast tumor cells, *Mol. Pharmacol.* 52 (1997) 373–379.
- [18] J. Botos, R. Smith, D.T. Kochevar, Retinoblastoma function is a better indicator of cellular phenotype in cultured breast adenocarcinoma cells than retinoblastoma expression, *Exp. Biol. Med.* 227 (2002) 354–362.
- [19] Q.S. Shao, Z.Y. Ye, Z.Q. Ling, J.J. Ke, Cell cycle arrest and apoptotic cell death in cultured human gastric carcinoma cells mediated by arsenic trioxide, *World J. Gastroenterol.* 11 (2005) 3451–3456.
- [20] L.C. Chiang, L.T. Ng, I.C. Lin, P.L. Kuo, C.C. Lin, Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells, *Cancer Lett.* (2005), available online.
- [21] B.G. Schlarb-Ridley, D.S. Bendall, C.J. Howe, Relation between interface properties and kinetics of electron transfer in the interaction of cytochrome *f* and plastocyanin from plants and the cyanobacterium *Phormidium laminosum*, *Biochemistry* 42 (2003) 4057–4063.
- [22] C.J. Sherr, The Pezcoller lecture: cancer cell cycles revisited, *Cancer Res.* 60 (2000) 3689–3695.