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Mitochondria-to-nucleus stress signaling induces phenotypic changes, tumor progression and cell invasion

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Recently we showed that partial depletion of mitochondrial DNA (genetic stress) or treatment with mitochondrial-specific inhibitors (metabolic stress) induced a stress signaling that was associated with increased cytoplasmic-free Ca^{2+} $[Ca^{2+}]_c$. In the present study we show that the mitochondria-to-nucleus stress signaling induces invasive phenotypes in otherwise non-invasive C2C12 myoblasts and human pulmonary carcinoma A549 cells. Tumor-specific markers cathepsin L and transforming growth factor β (TGFβ) are overexpressed in cells subjected to mitochondrial genetic as well as metabolic stress. C2C12 myoblasts subjected to stress showed 4- to 6-fold higher invasion through reconstituted Matrigel membrane as well as rat tracheal xenotransplants in Scid mice. Activation of Ca^{2+}-dependent protein kinase C (PKC) under both genetic and metabolic stress conditions was associated with increased cathepsin L gene expression, which contributes to increased invasive property of cells. Reverted cells with ~70% of control cell mtDNA exhibited marker mRNA contents, cell morphology and invasive property closer to control cells. These results provide insights into a new pathway by which mitochondrial DNA and membrane damage can contribute to tumor progression and metastasis.

Keywords: calcium-dependent PKC/cathepsin L expression/invasive phenotypes/mitochondrial membrane potential/stress signaling

Introduction

Since Warburg's initial hypothesis (Warburg, 1956), the role of mitochondrial dysfunction in tumorigenesis has been investigated extensively using multiple approaches (Shay and Werbin, 1987; Cavalli and Liang, 1998). Cytoplasm from tumor cells was shown to transfer tumorigenic property when fused with karyoplasts from normal cells, suggesting that unknown cytoplasmic factors may support the formation of malignant phenotypes (Jonasson *et al*., 1977; Howell and Sager, 1978; Giguere and Morais, 1981). Recent reports showing point mutations and deletions in mtDNA from a wide range of tumor cells (Horton *et al*., 1996; Polyak *et al*., 1998; Fliss *et al*., 2000; Yeh *et al*., 2000) have rekindled interest in Warburg's hypothesis. Studies with mtDNA-depleted tumor cells have yielded mixed results, ranging from minimal effects to possible cooperativity between the mitochondrial and nuclear genes in tumor growth (Giguere and Morais, 1981; Morais *et al*., 1994; Cavalli *et al*., 1997; Cavalli and Liang, 1998; Hofhaus and Gattermann, 1999). Currently there is increasing evidence that mitochondrial mutations and/or structural–functional abnormality are associated with various tumors (Shay and Werbin, 1987; Cavalli and Liang, 1998), although it is not clear whether the mitochondrial lesions are contributing factors in carcinogenesis or whether they simply arise as part of the secondary effects of cancer.

The mode of mitochondria-to-nucleus communication has been intensively studied in yeast ρ° cells, in which a number of nuclear genes including those of mitochondrial rRNA and *CIT2* (gene for peroxisomal citrate synthase) are overexpressed (Parikh *et al*., 1987; Liao *et al*., 1991). Overexpression of nuclear genes was also reported in ρ° avian and human cells (Marusich *et al*., 1997; Wang and Morais, 1997). Detailed mechanistic studies in yeast have demonstrated that upregulation of *CIT2* gene expression involves the activation and nuclear translocation of heterodimeric basic helix–loop–helix (bHLH) factors, Rtg1 and Rtg3, in response to mitochondrial stress (Liao and Butow, 1993; Sekito *et al*., 2000). It has been suggested that mitochondrial O_2 tension or oxidative stress may somehow be involved in the activation of cytoplasmic regulatory factor Rtg2 (Rothermel *et al*., 1995). Recently we described mitochondria-to-nucleus stress signaling in C2C12 myoblasts that were partially depleted of mtDNA or treated with mitochondrial inhibitors, conditions that disrupted mitochondrial membrane potential, $\Delta\psi_m$. Disruption of $\Delta\psi_m$ was accompanied by elevated cytosolic free $[Ca^{2+}]_i$ and activation of Ca^{2+}-responsive transcription factors and calcineurin (Biswas *et al*., 1999). Elevated Ca^{2+} and increased extracellular signal-regulated kinase 1 (ERK1) and ERK2 activity were also observed in PC12 pheochromocytoma cells treated with FCCP, a mitochondria-specific ionophore (Luo *et al*., 1997). Results emerging from these studies, therefore, demonstrate that mitochondrial stress signaling in different cell types modulates nuclear gene expression.

In continuation of these studies, here we show that mitochondrial stress signaling induces an array of nuclear genes including cathepsin L, transforming growth factor (TGFβ), mouse melanoma antigen (MMA) and others, which are well known markers for tumorigenesis. We describe a novel mechanism whereby damage to mtDNA and mitochondrial membrane signals a change in nuclear

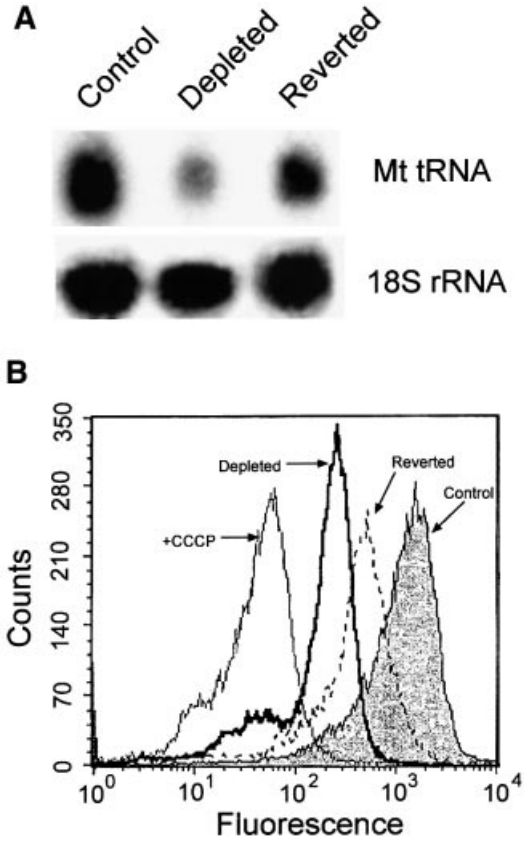


Fig. 1. Changes in mitochondrial membrane potential in mtDNA-depleted and reverted cells. (**A**) Mitochondrial $tRNA^{Leu}$ levels in control, mtDNA-depleted and reverted C2C12 myoblasts, determined by northern blot analysis of total RNA (20 μg in each lane) using ^{32}P-labeled DNA probe. The same blot was stripped and reprobed with 18S rRNA probe to assess the loading level. (**B**) Flow cytometry analysis of cells stained with mitochondrial-specific cationic dye, Mitotracker, as described in Materials and methods.

gene expression, leading to overexpression of marker genes for tumor progression. Genetic and metabolic stress, both of which affect $\Delta\psi_m$, convert the otherwise non-tumor-forming C2C12 myoblasts and non-invasive human lung carcinoma A549 cells to highly tumorigenic and invasive phenotypes.

Results

Effects of partial mtDNA depletion on mitochondrial membrane potential

In a recent study, we generated a series of C2C12 cell lines containing varying levels of mtDNA (20–50% of control cells) by ethidium bromide treatment for 40–50 growth cycles, followed by single cell cloning. mtDNA-depleted cells exhibited varying levels of mitochondrial transmembrane potential, $\Delta\psi_m$, elevated cytoplasmic free $[Ca^{2+}]_i$ and elevated nuclear genome-coded ryanodine receptor 1 (*RyR1*) mRNA (Biswas *et al.*, 1999). These processes were reversed to near normal cellular levels in a 'reverted cell line', whose mtDNA content was brought back to >70% of the control by growing mtDNA-depleted cells (20% mtDNA content) in normal medium, lacking ethidium bromide, for ~30 growth cycles. In the present study we used these cell lines for characterizing further the nature of mitochondrial stress signaling and its potential nuclear gene targets. The northern blot in Figure 1A shows the level of mitochondrial gene-coded $tRNA^{Leu}$ in control, mtDNA-depleted and reverted cells. The depleted cells show ~70% reduced $tRNA^{Leu}$, while the reverted cells show ~30% reduced RNA compared with control cells. The mitochondrial RNA levels directly reflect the cellular mtDNA contents. Figure 1B shows the extent of the staining of cells with mitochondrial-specific cationic dye, Mitotracker, analyzed by flow cytometry. Results show that control cells exhibit maximum emission of 2×10^3, while mtDNA-depleted cells show a reduced emission of $\sim 2 \times 10^2$. The reverted cells show an intermediate intensity of 5×10^2. Cells treated with CCCP for 1 h show the least intensity of $\sim 5 \times 10^1$, signifying most disruption of $\Delta\psi_m$. In confirmation of our previous results with confocal microscopy, these results show a more severe disruption of $\Delta\psi_m$ in cells with a higher level of mtDNA depletion.

Table I. Differential expression of nuclear genes in mtDNA-depleted C2C12 cells

Genes identified	mRNA levels (fold of control cells)	
	Depleted	Reverted
RyR1	18.0	3.0
Calsequestrin	4.1	1.1
Calreticulin	2.5	1.2
Cathepsin L	5.0	2.0
Mouse melanoma antigen	16.0	2.5
PEP carboxykinase	3.0	1.5
Two novel genes with no homology to published sequence	4.0–5.3	2.0–3.0
TGFβ1	5.0	1.1
Insulin receptor substrate 1	0.5	0.7
mtDNA transcripts	0.2	0.7–0.9

Primer extension products showing increased or decreased RNA levels in mtDNA-depleted cells were PCR amplified and sequenced to determine the identity of genes. The DNA fragments were also used as probes in northern blot hybridization with RNA from control, depleted and reverted cells. The RNA levels are expressed as fold of control cell level, considered to be 1.

Nuclear gene targets of mitochondrial stress signaling

To gain insight into the nature of nuclear gene targets of the mitochondrial stress signaling, we carried out differential display of RNA from control, mtDNA-depleted and reverted cells. We observed that 96 display products were either overexpressed or suppressed compared with control cell products. Of these, 35 display products were analyzed by DNA sequencing and northern hybridization; 17 products, listed in Table I, were positive. RNA from three different depleted cell lines reproducibly showed 2.5- to 18-fold increased mRNA for RyR1 Ca^{2+} channel, calreticulin, calsequestrin, MMA, cathepsin L, TGFβ and phosphoenol pyruvate (PEP) carboxykinase, in addition to two novel genes with no homology to sequences in the database (Table I). We also observed uniformly reduced mtDNA-encoded, as well as nuclear-encoded cytochrome oxidase VIIa and insulin receptor

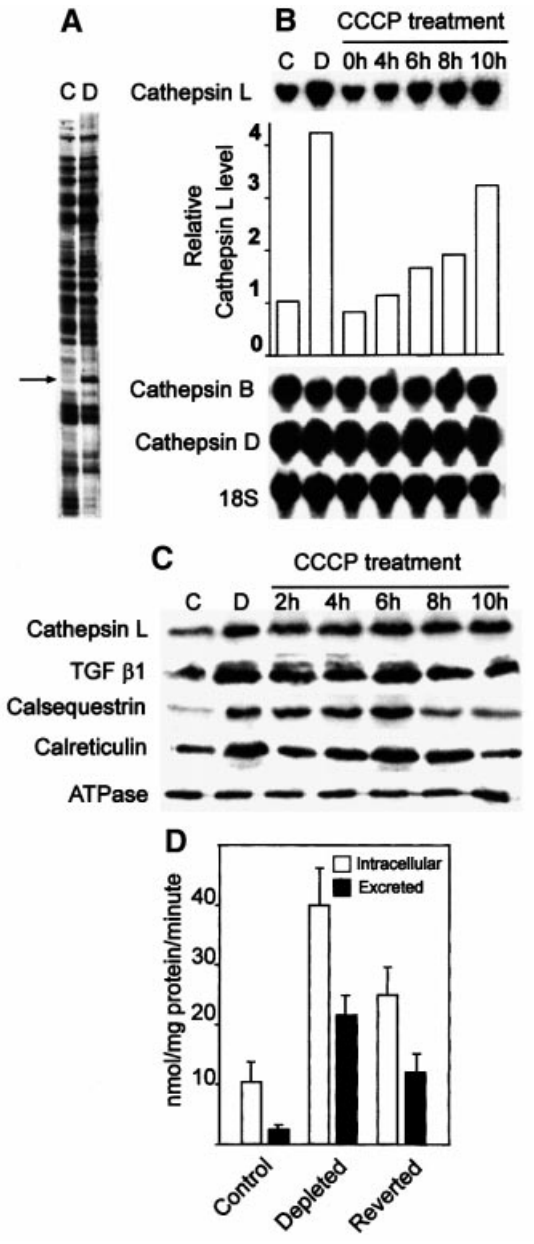


Fig. 2. Induction of cathepsin L during mitochondrial genetic and metabolic stress. (**A**) Differential display pattern showing induced level of cathepsin L mRNA in mtDNA-depleted cells. (**B**) Northern blots using cathepsin L cDNA probe (upper panel) and cathepsin B and D cDNA probes (lower panels) from control C2C12 cells (C) and mtDNA-depleted cells (D), and also cells treated with CCCP (25 μM) for the indicated time. In all panels 20 μg RNA was hybridized with ^{32}P-labeled DNA probes under standard conditions. The blots were reprobed with 18S rRNA probe to ensure the RNA loading. (**C**) Immunoblot analysis of total cell extracts (50 μg protein in each case) from control and treated cells as indicated at the top. The blots were probed with indicated antibodies (1:1000 dilution) and developed using the Superglo kit from Pierce (Rockford, IL). The blots were reprobed with antibody against Na^+/K^+ ATPase to determine the protein loading level. The RNA and protein blots were quantified using a BioRad radiometric imager. (**D**) The level of intracellular and extracellular cathepsin L activity was assayed in total cell extract or concentrated culture fluid as described in Materials and methods. The values represent mean ± SEM of four experiments.

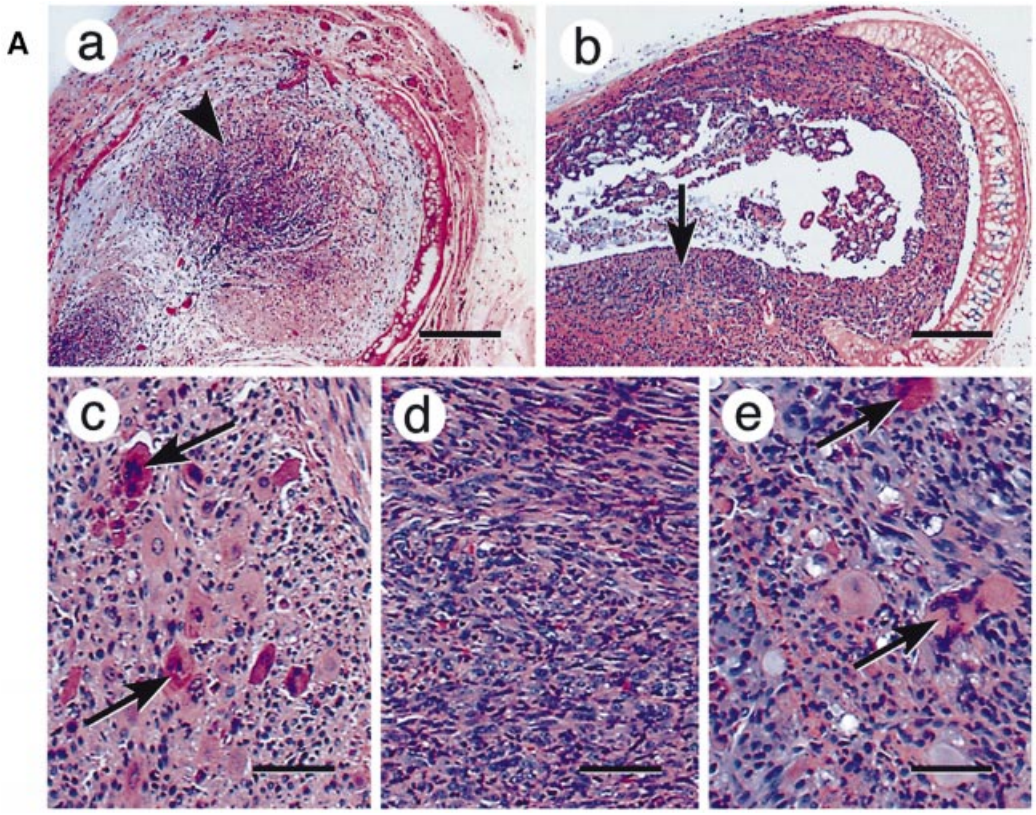
substrate 1 mRNAs. The altered mRNA levels for all the listed genes was due to mitochondrial genetic stress, since they were restored to near normal levels (1- to 2.5-fold of control cells, Table I) in reverted cells with 70% restored mtDNA and a steeper membrane potential (Figure 1).

The effects of mitochondrial stress on the expression of well known tumor marker genes cathepsin L and TGFβ were investigated using CCCP, a mitochondrial-specific ionophore, as a model metabolic inhibitor. The gel pattern in Figure 2A shows a 6- to 8-fold increased differential display product, which was found to be cathepsin L specific. The northern blot in Figure 2B (top panel, first two lanes) shows a >4-fold higher level of cathepsin L mRNA in mtDNA-depleted cells compared with control C2C12 cells. Additionally, cathepsin L mRNA is increased 2- to 3-fold in cells treated with CCCP for 6–10 h (top panel of Figure 2B, last five lanes). However, cathepsin B and D mRNA levels remained the same in cells subjected to both types of stress, suggesting specificity of nuclear gene targets affected by the stress signaling. Consistent with the mRNA level, the western blot in Figure 2C shows a 3- to 5-fold increased cathepsin L protein in both mtDNA-depleted cells and cells treated with CCCP. The level of TGFβ protein increased by ~4.5-fold in cells subjected to both genetic and metabolic stress. In further support of the differential display data in Table I, the levels of calsequestrin and calreticulin were increased 4-fold in mtDNA-depleted cells, as well as in CCCP-treated cells. For unknown reasons, the level of calsequestrin was reduced to near normal levels after 8 h of CCCP treatment. The levels of plasma membrane ATPase used as a loading control, on the other hand, did not change due to mitochondrial stress. Unpublished results (G.Biswas, H.Anandatheerthavarada and N.Avadhani) also show that mtDNA-depleted C2C12 cells contain increased BCl2 levels and show resistance to apoptosis. These results suggest that mitochondrial genetic stress signaling regulates the expression of nuclear genes involved in a wide range of cellular locations and functions, in addition to those targeted to mitochondria.

Elevated secretion of matrix protease cathepsin L is an important marker for invasive tumors (Cuvier *et al*., 1997; Kim *et al*., 1998). We therefore determined the levels of cathepsin L secreted in the culture media of normal and mtDNA-depleted cells. Figure 2D shows that control C2C12 cells secreted a relatively small amount of cathepsin L, while mtDNA-depleted cells secreted ~10-fold more than control cells. The reverted cells secreted significantly lower levels of cathepsin L, which amounted to ~5-fold higher than control cells. These results show that both intracellular as well as the excreted cathepsin L level is increased in mtDNA-depleted cells.

Effects of mitochondrial stress on tumor progression

Overexpression of procathepsin L converted human melanoma cells from a non-metastetic to highly metastetic state (Denhardt *et al*., 1987; Yagel *et al*., 1989; Welch *et al*., 1990; Frade *et al*., 1998; Kirschke *et al*., 2000), while its inhibition by antisense RNA reversed the invasive property of tumor cells (Kirschke *et al*., 2000). We therefore tested the role of mitochondrial stress on tumor progression using a xenotransplantation assay (Momiki *et al*., 1991), which involves growth of tumor cells inside de-epithelialized rat tracheas, transplanted



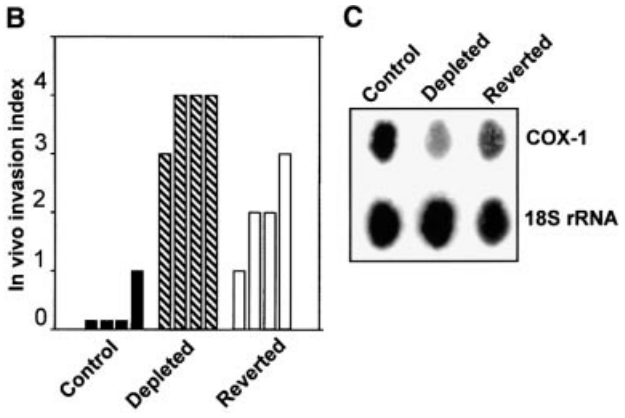


Fig. 3. Induced tumorigenicity of mtDNA-depleted C2C12 cells *in vivo* in Scid mice. (**A**) Hematoxylin–eosin stained sections of xenotransplants are shown. Control cells (a) that are growing inside the former tracheal lumen are shown by an arrowhead (invasion level 0). Panel b shows a representative *in vivo* growth pattern of mtDNA-depleted cells in a tracheal transplant. Note that the cells are growing inside the trachea as well as invading the external tracheal wall, as indicated by the arrow (invasion level 3–4). Panels c, d and e show the cellular differentiation pattern of control, mtDNA-depleted and reverted cells, respectively. The giant multinucleated rhabdomyoblasts (shown by arrows) are clearly seen in the control (c) and reverted cells (e). Such multinucleated cells are not seen in mtDNA-depleted cells (d). Bars in a and b represent 800 μm and those in c, d and e represent 100 μm. (**B**) The invasion levels of control, depleted and reverted cells from four different double blind assays are presented as bar diagrams. (**C**) Mitochondrial DNA contents of cells recovered from the representative sections of tracheal transplants as in (A). Total cell DNA (5 μg each) were subjected to Southern blot hybridization with mitochondrial-specific cytochrome oxidase I DNA probe. The blot was stripped and rehybridized with labeled probe for 18S rRNA probe to determine the loading level.

subcutaneously into Scid mice (Figure 3A). Histological examination of transplants showed that control C2C12 cells (Figure 3A) grew mostly in the middle of the tracheal transplant with no detectable invasion of the tracheal wall. mtDNA-depleted cells, on the other hand, exhibited vastly increased invasiveness (Figure 3B). In the latter case, cells destroyed the tracheal wall and its cartilage, spreading into the host tissue.

We used an arbitrary invasion scale based on histopathological examination of xenotransplants as described (Momiki *et al*., 1991). According to the scheme, level 0 is no invasion, where cells remain confined to the luminal ring; level 1 is minimal invasion of the subepithelial tissue; level 2 is moderate invasion, where cells reach the pars membranes; level 3 is marked invasion, where cells are outside the tracheal wall, covering and infiltrating the adventitia; and level 4 is maximal invasion, where cells are in the adventitia growing in large nodules or masses larger than the intratracheal tumor. Results of four independent double blind assays in Figure 3B show that the control cells showed an invasion level of 0–1, the mtDNA-depleted cells showed the highest level of 4, in three out of four cases, while the reverted cells showed a medium range of 1–3. Although not shown, three different reverted cell lines showed uniformly reduced invasion.

At higher magnification, the xenotransplanted control cells (Figure 3A, c) were seen to contain giant multinucleated rhabdomyoblasts, which are characteristic of

differentiated skeletal myocytes. The mtDNA-depleted cells were poorly differentiated with no multinucleated structures (d). The reverted cells (e), on the other hand, showed a number of multinucleated structures similar to that seen in normal cells. It is therefore remarkable that reverted cells not only show an altered morphology closer to control untreated cells, but also exhibit a marked reduction in their invasive behavior.

Since the reverted cells showed a significantly lower invasive behavior compared with mtDNA-depleted cells, the mitochondrial DNA content of the various cell types during the 5 weeks of growth in the xenotransplants was an important issue. This question was addressed by assessing the mtDNA levels in the total DNA isolates from companion sections of xenotransplants shown in Figure 3A. A large majority of cells from these tracheal sections (>80%) were myoblasts, and 15–20% were fibroblasts from the host tissue. Results of Southern hybridization in Figure 3C reveal that explants of depleted cells show ~40% mtDNA content, while those of reverted cells show ~70% of control, suggesting the mtDNA levels change only marginally during the time frame of xenotransplantation assay.

Role of cathepsin L in mitochondrial stress-induced invasion

The role of cathepsin L and TGFβ in mitochondrial stress-induced invasiveness was investigated further using an *in vitro* Matrigel assay system (Yagel *et al*., 1989). This system measures the extent of cell migration through reconstituted biologically active basement membrane matrix, coated on microporous membrane. From Figure 4A it can be seen that mtDNA-depleted cells show >5-fold higher invasion, while cells treated with CCCP for 5 h show a 2.5-fold higher invasion as compared with untreated cells. Interestingly, a peptide inhibitor of cathepsin L reduced the invasive behavior of cells under both types of stress conditions in a concentration-dependent manner, while antibody to TGFβ had no effect. Cyclosporin, an immunosuppressive drug, was recently shown to induce the invasiveness of A549 human pulmonary carcinoma cells, through the TGFβ pathway (Welch *et al*., 1990; Hojo *et al*., 1999). In C2C12 cells, cyclosporin induced 2-fold higher invasion, which was not reversed even by 60 μM cathepsin L inhibitor peptide (see Figure 4A). These results show that induced cathepsin L gene expression is associated with tumor invasion during both mitochondrial genetic and metabolic stress conditions.

The generality of the mitochondrial stress-induced tumor invasion was tested using human pulmonary A549 cell line, which has been shown to be non-invasive under *in vivo* and *in vitro* conditions (Frade *et al*., 1998). This cell line was recently used as a model system to study cyclosporin-mediated tumorigenesis (Hojo *et al*., 1999). The state of mitochondrial membrane potential in A549 cells treated with CCCP was assessed by the extent of staining with Mitotracker. The western blot of whole-cell extracts in Figure 4B shows that the level of cathepsin L is induced 3- to 4-fold in cells treated with CCCP for 4–10 h in comparison with untreated normal cells. Figure 4C shows that 5 h of CCCP treatment induced the invasiveness to ~2.5-fold of control, which was reduced to 1.5-fold level by treatment with cathepsin L inhibitor peptide. In support of a recent study (Hojo *et al*., 1999), cyclosporin treatment also induced the invasive behavior of A549 cells by ~3-fold, which was refractory to treatment with cathepsin L inhibitor peptide. Although not shown, the cyclosporin-induced invasion, but not that induced by mitochondrial stress, was partially reversed by monoclonal antibody to TGFβ.

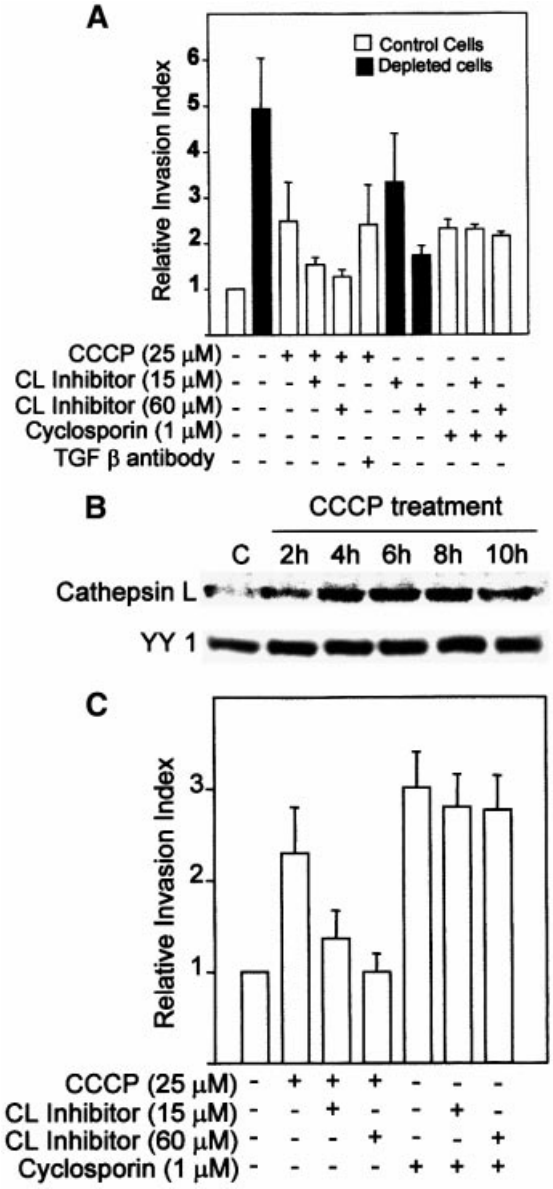


Fig. 4. Mitochondrial stress-induced cell invasion in C2C12 myoblasts and A549 lung carcinoma cells. *In vitro* Matrigel assays were carried out using ^{3}H-labeled cells as described in Materials and methods. Treatment with indicated levels of CCCP and cyclosporin was carried out for 5 h, and other treatments were as described in Materials and methods. (**A**) *In vitro* invasion of C2C12 cells in Matrigel chambers. (**B**) Effects of CCCP on the induction cathepsin L protein in A549 cells. Immunoblot analysis of whole-cell extracts (50 μg protein each) was carried out with antibody to human cathepsin L (Santacruz Biotech., 1:1000 dilution). The blot was also reprobed with antibody to ubiquitous transcription factor YY1 as a loading control. (**C**) *In vitro* Matrigel invasion with treated and untreated A549 cells. The values represent mean ± SEM of four independent experiments.

Mechanism of stress-induced upregulation of cathepsin L gene expression

The murine cathepsin L promoter (Troen *et al*., 1991) cloned in a luciferase reporter vector, pGL3, was used to investigate the mechanism of mitochondrial stress-mediated transcription activation. Figure 5A shows the protein binding motifs of the promoter based on sequence analysis (Troen *et al*., 1991). In transient transfection assays, the promoter DNA construct (Figure 5B) showed a 5.5-fold higher transcription activity in mtDNA-depleted cells compared with control C2C12 cells. The reverted cells showed ~1.7-fold higher activity than the control cells. Treatment of control C2C12 cells for 5 h with CCCP also resulted in a 3- to 4-fold induced transcription of the promoter construct. The increased transcription in mtDNA-depleted cells was due to increased intracellular Ca^{2+}, since the activity was sensitive to the calcium chelator BAPTA/AM. Transcription of the promoter construct in mtDNA-depleted cells was selectively sensitive to added PKC inhibitor H7, but not to tyrosine kinase inhibitor, herbimycin A. These results are in line with the reported effects of kinase inhibitors on cathepsin L mRNA levels in intact cells (Atkins and Troen, 1995).

Figure 5C shows PKC activity assayed based on the extent of inhibition by a pseudosubstrate inhibitor peptide that is specific for Ca^{2+}-dependent PKC α, β and γ isoforms. Results show that PKC activity is induced 2-fold in mtDNA-depleted cells, which returns to near control cell levels (1.1- to 1.2-fold of control) in reverted cells. A comparable change in the total Ca^{2+}-sensitive kinase activity is also observed in these cells (Figure 5C). Thus, there is close correlation between the PKC activity, transcriptional activity of the promoter and the cathepsin L mRNA levels in the three cell types. A possible link between these two factors was investigated by co-transfection with Egr-1 cDNA and isoform-specific PKC cDNAs. Additionally, the role of Egr-1 in transcription activation of cathepsin L promoter was tested by using promoter constructs with point mutations targeted to the Egr-1 binding site. As shown in Figure 5D, co-transfection with Egr-1 cDNA alone or PKC cDNAs alone had no effect on the activity of the wild-type as well as Egr-1 mutant promoter constructs in control C2C12 cells. A combination of Egr-1 and PKC α, β or γ cDNAs, however, yielded ~4-fold higher transcription activities with the wild-type promoter construct but not with the mutant construct. Similarly, co-transfection with Fli cDNA, a close relative of Egr-1 factor with 90% sequence conservation had no stimulatory effect even in the presence of PKC α, β or γ cDNAs.

The levels of Egr-1-specific DNA binding proteins in the nuclear extracts from various cell types were examined by gel mobility shift assays. As shown in Figure 6A, nuclear extract from control cells formed two protein complexes marked as complex I and II. The nuclear extract from mtDNA-depleted cells formed these same complexes at 4- to 5-fold higher intensity, in addition to a slower migrating complex. The latter complex, but not complexes I and II, was supershifted by Egr-1 antibody. All of the complexes with control and depleted nuclear extract were competed by unlabeled excess Egr-1 DNA. Furthermore, nuclear extract from reverted cells showed

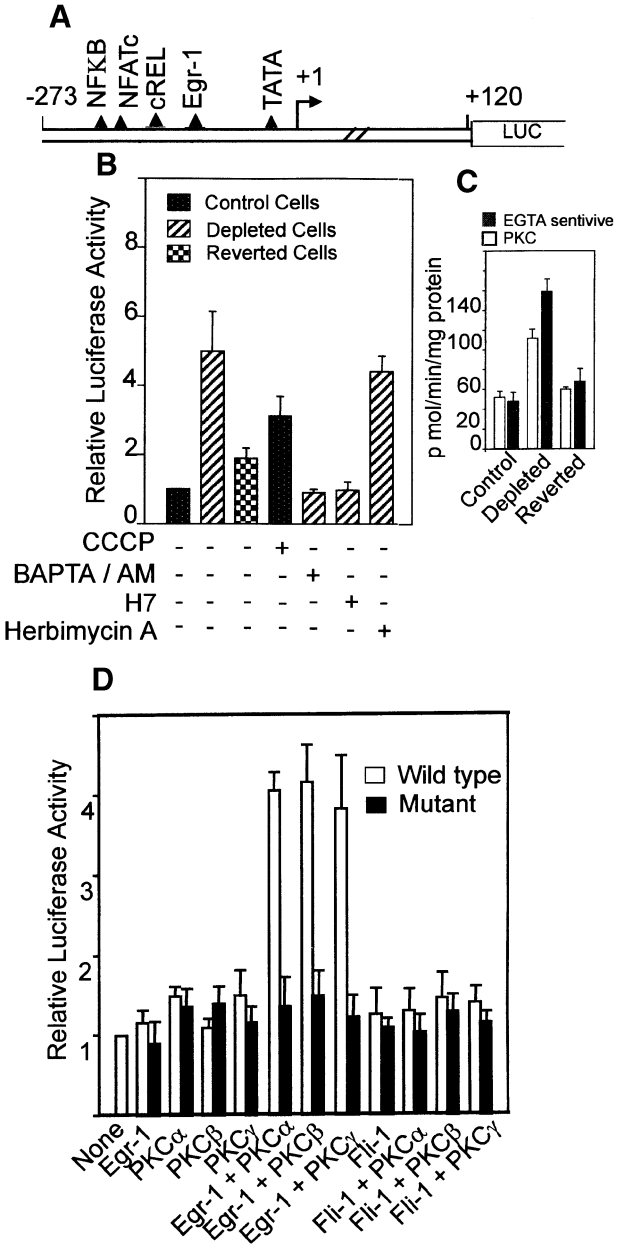


Fig. 5. Mechanism of activation of cathepsin L promoter by mitochondrial stress. (**A**) Structure of murine cathepsin L promoter and potential protein binding motifs based on nucleotide sequence. (**B**) Transcriptional activity of promoter construct in pGL3 reporter vector (Promega, Madison, WI). Details of transfection with reporter and renila luciferase as internal control and analysis of luciferase activity were as described in Materials and methods. Various agents as indicated were added to transfected cells also as described in Materials and methods. (**C**) PKC activity was assayed based on the level of inhibition with a PKC α, β and γ-specific pseudosubstrate inhibitor peptide. Ca^{2+}-sensitive activity represents the level of inhibition with 5 mM EGTA. (**D**) Effects of coexpression with Egr-1 and PKC isoform-specific cDNAs on the transcriptional activity of the cathepsin L promoter in control C2C12 cells. Values represent the average ± SEM of 4–6 transfection assays in all cases.

reduced levels of antibody-reactive complex. These results suggest that Egr-1 is indeed induced in mtDNA-depleted cells. The nature of similarly migrating complexes I and II obtained with all three nuclear extracts remain unclear, although they may represent related protein(s) with similar DNA-binding specificity. Although not shown, the levels

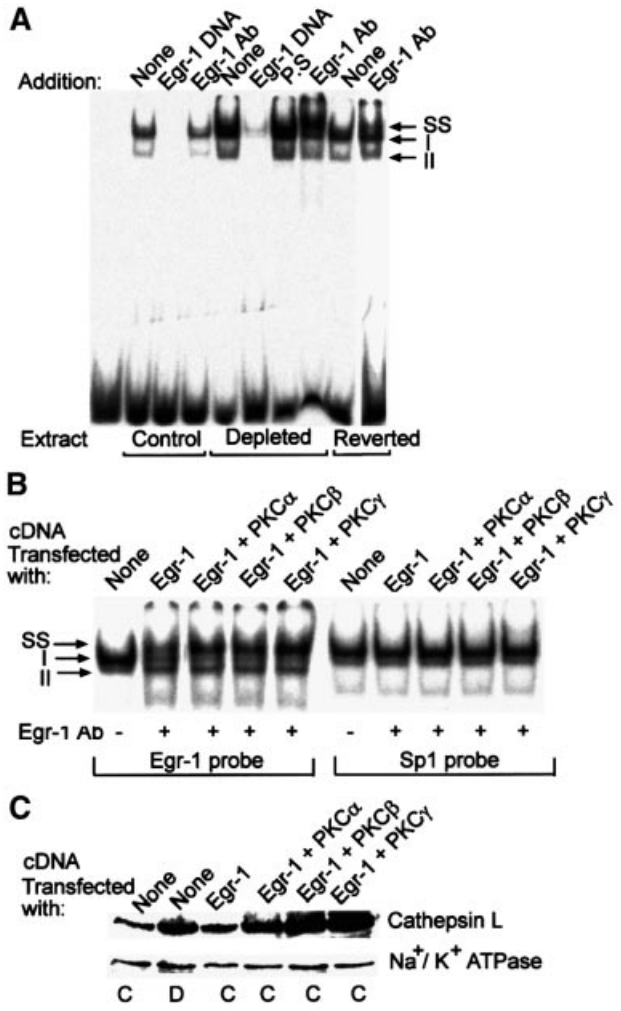


Fig. 6. Role of Ca^{2+}-activated PKC on the activation of Egr-1 and endogenous cathepsin L gene expression. (**A**) Levels of Egr-1 DNA binding factors in the nuclear extracts from control, depleted and reverted (R) cells by gel mobility shift analysis (P.S, pre-immune serum; SS, supershifted). (**B**) Gel shift patterns with nuclear extracts from cells transfected with Egr-1 alone, and with PKCα, β or γ cDNAs. In each case, 7.5 μg of protein was used for DNA binding with Egr-1 DNA (left panel) and Sp1 DNA (right panel). (**C**) The western blot analysis of whole-cell extracts (50 μg each) from control C2C12 cells transfected with Egr-1 cDNA alone and with either PKC α, β or γ cDNAs. The blot was co-developed with antibody to cathepsin L and antibody to Na^+/K^+ ATPase as a loading control (C, control extracts; D, depleted extract).

of ubiquitous factors Sp1 and YY-1 were similar in nuclear extracts from all three cell types.

To understand further the role of PKC isoforms on the activity of Egr-1, nuclear extracts from control cells transfected with Egr-1 cDNA alone and in combination with PKCα, β or γ cDNAs were analyzed by gel shift with Egr-1 and Sp1 DNA probes (Figure 6B). Results show that nuclear extract from Egr-1 cDNA-transfected cells formed a complex that was marginally supershifted with Egr-1 antibody, while nuclear extracts from cells transfected with Egr-1 and any of the three PKC cDNAs yielded prominent supershifting complexes. Although not shown, transfection with PKC cDNAs alone did not yield antibody supershifting complex. The level of Sp1 DNA binding complex remained the same in these cells. Additionally, complexes with Sp1 DNA were not supershifted with Egr-1 antibody, suggesting specificity. These results demonstrate that all three Ca^{2+}-activated PKC isoforms increase the DNA binding ability of Egr-1. The immunoblot in Figure 6C shows that transfection conditions that increased the formation of antibody supershifting complexes also increased the cellular cathepsin L protein levels by 4- to 5-fold over control cells. Although not shown, C2C12 cells co-transfected with Egr-1 and any of the three Ca^{2+}-specific PKC cDNAs showed 5- to 6-fold higher invasiveness through the Matrigel membrane system. These results therefore provide direct evidence for the role of Ca^{2+}-dependent PKC in the transcription regulation of cathepsin L gene expression. Results also indicate that the Ca^{2+}-specific PKC effects are mediated through Egr-1 factor binding to the cathepsin L promoter.

Discussion

Mitochondrial DNA in cultured cells as well as in intact animals has been shown to be a direct and preferential target of attack by varied carcinogens (Allen and Coombs, 1980; Backer and Weinstein, 1980; Niranjan *et al*., 1982). Mitochondria are important cellular sites for the production of reactive O_2 species (ROS) under different physiological and pathological conditions (Kehrer, 1993; Lenaz, 1998). These observations, coupled with the occurrence of DNA excision repair in mitochondria (Pinz and Bogenhagen, 1998), suggest that mtDNA is a likely target of mutational damage under both oxidative and chemical stress conditions. In support of this view, a variety of homoplasmic, as well as heteroplasmic mtDNA mutations have been reported in a number of human tumors (Horton *et al*., 1996; Polyak *et al*., 1998; Fliss *et al*., 2000; Yeh *et al*., 2000). It is, however, unclear whether these mutations are simply the consequence of increased oxidative stress, known to occur during tumor progression, or whether they have any direct role in the progression of cancer. Results presented in this paper provide a mechanistic insight into a pathway of cancer progression and tumor invasion resulting from mitochondrial DNA and/or membrane damage.

While investigating the potential nuclear gene targets of the mitochondria-to-nucleus stress signaling (Table I), we found that some of the genes implicated in tumor progression are also overexpressed in C2C12 myoblasts subjected to mitochondrial genetic stress. Growth factor receptor, TGFβ and extracellular matrix protease cathepsin L are among the well known tumor markers induced in many invasive cancers (Denhardt *et al*., 1987; Yagel *et al*., 1989; Welch *et al*., 1990; Frade *et al*., 1998; Hojo *et al*., 1999; Kirschke *et al*., 2000). Our results show that inhibition of cathepsin L, but not TGFβ, causes a partial reversal of mitochondrial stress-induced invasive behavior, both in C2C12 myoblasts and lung carcinoma A549 cells. These results show that cathepsin L gene expression is important in mitochondrial stress-induced *in vitro* invasion and is likely to be important in *in vivo* invasion as well.

Results of this study also show that, in addition to the Ca^{2+}-dependent calcineurin pathway described before, the Ca^{2+}-dependent PKC pathway is also activated as part of mitochondrial genetic and metabolic stress signaling.

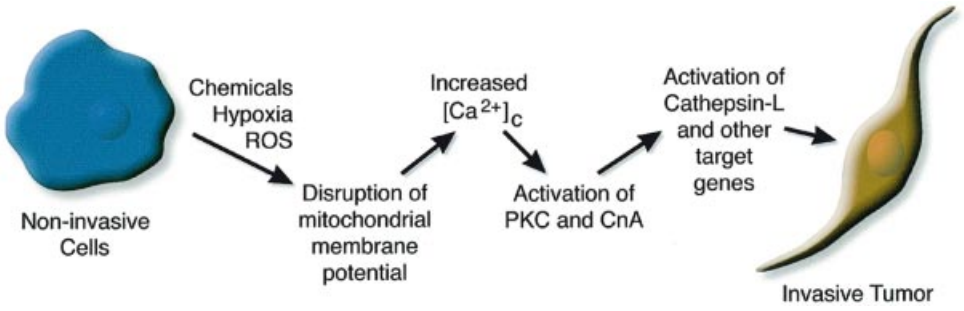


Fig. 7. A model for the mitochondrial genetic and metabolic stress-mediated tumor invasion.

A recent study showed that mitochondrial oxidative stress through overproduction of H_2O_2 induced the Jun kinase pathway (Nemoto *et al*., 2000). These results are consistent with our previous study, which showed that mitochondrial genetic and metabolic stress induced JNK (c-Jun NH_2-terminal kinase)-dependent activation and associated nuclear localization of activated transcription factor 2 (ATF2) (Biswas *et al*., 1999). In the present study we demonstrate that the ability of mtDNA-depleted cells and CCCP-treated cells to support higher transcription activity of the cathepsin L promoter is directly related to PKC activation. Mutational analysis together with DNA–protein binding also suggest the importance of Egr-1 factor for promoter activity. Egr-1, a member of immediate early Jun/Fos family genes, was suggested to be activated by phosphorylation (Cao *et al*., 1990; Huang and Adamson, 1994). Rat cathepsin L gene is reported to be activated by Egr family proteins (Ishidoh *et al*., 1997). Results of this study suggest for the first time a direct link between Ca^{2+}-dependent PKC and the DNA-binding activity of Egr-1 in modulating cathepsin L gene expression. Similar PKC-mediated activation of other members of immediate early family transcription factors has been reported (Grover-Bardwick *et al*., 1994). Consistent with this possibility, murine Egr-1 contains four consensus motifs for PKC, although the precise phosphorylation site critical for activation and the precise effects of phosphorylation on DNA binding remain unclear. Transcription activation of cathepsin L gene expression is highly dependent on Egr-1 binding, since other distant members of this family, Jun/Fos, and the very close relative Fli are unable to induce the transcription of the promoter construct or the endogenous Egr-1 gene.

A remarkable observation of this study is that mitochondrial stress-induced phenotypic changes of invasive behavior, patterns of gene expression and ability to differentiate into multinucleated rhabdomyocytes are reversed, albeit partly, in reverted cells containing 70% of control cell mtDNA content. A partial recovery of invasiveness in reverted cells under both *in vivo* and *in vitro* conditions is consistent with their higher level of marker gene expression and Ca^{2+}-dependent PKC activity compared with control C2C12 cells. Nevertheless, reduced invasive behavior in cells with reduced stress signaling provides a direct link between these two processes. Although not shown, Ca^{2+}-dependent PKC activity and Egr-1-specific DNA binding activity are returned to near normal levels (1.2- to 1.3-fold of control cells) in three different reverted cell lines containing ~70% of mtDNA content.

Acquired resistance to apoptosis in response to various apoptotic stimuli or cytotoxic drugs is the hallmark of many invasive tumors. In keeping with these, mtDNA-depleted C2C12 myocytes and also CCCP-treated A549 cells that show high invasive behavior are resistant to apoptosis in response to varied agents including staurosporine, etoposide and CCCP (G.Biswas, G.Amuthan and N.Avadhani, unpublished results). A close association between disrupted $\Delta\psi_m$ and release of cytochrome *c* and procaspases has been noted in many studies (Kluck *et al*., 1997; Yang *et al*., 1997). However, some recent studies show that the release of cytochrome *c* and procaspases is not directly dependent on mitochondrial membrane depolarization (Bossy-Wetzel *et al*., 1998). Similarly, membrane depolarization alone appears insufficient for induction of apoptosis, since mtDNA-depleted osteosarcoma cells with altered $\Delta\psi_m$ and reduced ATP synthesis are more resistant to apoptotic stimuli than normal cells (Dey and Moraes, 2000). In support of these observations, recent results show 3- to 4-fold higher Bcl2 in mtDNA-depleted C2C12 cells and also A549 lung carcinoma cells, which might be the basis for the observed resistance of these cells to apoptosis by CCCP treatment (G.Biswas, H.Anandatheerthavarada and N.Avadhani, unpublished results).

A model for the proposed mitochondrial stress-induced cancer progression is presented in Figure 7. Although a complete spectrum of nuclear gene targets affected by this stress signaling and their functional significance remain unclear, results in Table I demonstrate the overexpression of putative tumor marker genes. We propose that ROS, activated carcinogens, various drugs and anti-viral/anti-tumor agents (Shigenaga *et al*., 1994; Sokolove, 1994; Melov *et al*., 1999) that affect mitochondrial genetic and membrane systems may induce mitochondrial stress signaling. The stress signaling may therefore be a critical factor in the ability of some cells within a tumor mass to acquire invasive phenotypes and undergo morphological changes leading to tumor progression and metastasis. In summary, mitochondrial dysfunction and the resulting alterations in nuclear gene expression through mitochondria-to-nucleus stress signaling might be an important factor in cancer progression and tumor cell metastasis. The mitochondrial stress signaling described in this and our previous study (Biswas *et al*., 1999) may also help explain mechanistic details of various degenerative diseases

associated with mitochondrial dysfunction, in addition to pathophysiological processes of myogenic differentiation (Rochard *et al*., 2000) and impaired insulin secretion (Tsuruzoe *et al*., 1998) by mtDNA-depleted cells. Finally, mitochondrial stress-induced cathepsin L expression and tumor invasion appear to be a common phenomenon in many cell types, since CCCP treatment induced the cathepsin L mRNA level and invasive property in various established tumor lines, including PC12 pheochromocytoma, C6 glioma, HepG2 cells and squamous cell carcinoma SCC9 and SCC12.

Materials and methods

Cell lines and culture

Murine C2C12 skeletal myoblasts (ATCC CRL1772) and human pulmonary carcinoma A549 cells (ATCC CCL 185) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Depletion of mtDNA by ethidium bromide treatment and isolation of C2C12 clone 2 with 20% of control cell mtDNA content was as described before (Biswas *et al*., 1999). Reverted cells represent clone 2 grown for 17 division cycles in the absence of ethidium bromide and contain 70% of control cell mtDNA. To ensure steady mtDNA levels, aliquots of the same cell isolate were used in all experiments and the mtDNA contents were routinely assayed in each experiment.

Flow cytometry

Mitotracker green (40 μM, Molecular Probes, Inc., Eugene, OR), diluted in serum-free DMEM (pre-warmed to 37°C), was added to CCCP-treated (20 μM, 1 h) and untreated cells in fresh medium, and incubated for 45 min with the dye. The adhered cells were washed twice with cold phosphate-buffered saline (PBS) and suspended in 1 ml PBS for analysis using a Becton Dickinson FACSCalibur Flow Cytometer. Mitotracker green was excited at 488 nm and fluorescence was detected at 525 nm.

Differential display

Differential display of RNA was carried out essentially as described (Liang and Pardee, 1992). Total RNA (400 ng each) from control and mtDNA-depleted C2C12 cells were reverse transcribed and the primer extension products were amplified using primers supplied in a kit from Display Systems Biotech (Vista, CA). The primer extension products showing different intensities on 8% sequencing gels were amplified and the PCR products were cloned in pCR2 vector (Invitrogen, Carlsbad, CA), sequenced and used as probes for northern analysis. Northern blot hybridization was carried out using ^{32}P-labeled DNA probes and 20 μg RNA under standard conditions.

In vitro invasion assay

The *in vitro* invasion assays were carried out as described previously (Yagel *et al*., 1989). The Matrigel invasion chambers were prepared at 1:2 dilution of Matrigel (Beckton Dickinson, Belford, MA) according to the manufacturer's protocol. Cells (5×10^4), uniformly labeled with [^{3}H]dU (American Radiochemicals Inc.) for 24 h, were loaded on top of the Matrigel layer. After incubation for 24–72 h at 37°C, non-invading cells and the Matrigel layer were quantitatively removed and the microporous membrane containing the invaded cells was counted in a scintillation counter to determine the extent of invasion. While testing the inhibitor or antibody-treated cells, the Matrigel also contained the appropriate amounts of inhibitors or antibodies.

In vivo xenotransplantation assay

Assays were carried out essentially as described before (Momiki *et al*., 1991). Briefly, 5×10^5 cells in 100 μl of tissue culture medium were seeded into the lumen of de-epithelialized rat tracheal tubes and sealed at both ends. The sealed tubes were transplanted into dorsal mouse subcutaneous tissue (Momiki *et al*., 1991). Transplants (4–8 for each cell type) were recovered after 5–6 weeks, cross-sectioned into 3 mm thick rings, fixed in 10% neutral buffered formaldehyde (NBF) and embedded in paraffin. The paraffin blocks were sectioned and stained with hematoxylin and eosin.

Cathepsin L activity

Cathepsin L activity was determined as described (Sheahan *et al*., 1989) using the synthetic substrate benzyl-phenylalanine-arginine-4-methoxy-2-naphthylamide (Z-Phe-Arg-MNA) (Enzyme Systems products, Dublin, CA). Total cell lysates or concentrated culture medium (~150 μg of protein) were assayed for cathepsin L activity in 50 μl of reaction mixture containing 0.1 M morpholinethanesulfonic acid (MES) buffer pH 3.5, 1 mM Z-Phe-Arg-MNA, 1 mM EDTA, 1 mM dithiothreitol, at 37°C for 10 min. The reaction was terminated by addition of 50 μl of 1 N HCl containing 2% Triton X-100. Fast blue (*o*-dianisidine tetrazotized from Sigma Chemicals Co., St Louis, MO) was added, the color developed for 15 min and OD520 determined. Culture medium was concentrated by filtration through Ambion Filtration system using 10 kDa cut-off membranes.

Assay for PKC activity

The PKC activity was measured using the PKC Assay System kit from Life Technologies (Grand Island, NY), which employs a PKC-specific peptide substrate and a pseudosubstrate inhibitor specific for Ca^{2+}-dependent PKC isoforms. The Ca^{2+}-sensitive kinase activity was assayed by adding 5 mM EGTA to the assay mix. PKC enzyme was partially purified by chromatography on DEAE–Sephacel microcolumns by a protocol described in the kit and assays were carried out in 50 μl volumes using 1 μCi of [γ-^{32}P]ATP.

Transcription analysis of promoter constructs

Cathepsin L promoter DNA (sequence –221 to +47) was amplified from the mouse genomic DNA based on the published sequence (Troen *et al*., 1991) and cloned in pGL3 vector (Promega, Madison, WI). Mutations at the Egr-1 binding site were targeted by substituting the two GG residues from the Erg-1 binding site with TA residues (wild type: CGGGGC-GGGGGCGGGC; mutant: CGGGGCTAGGGCTAGC) by primer-mediated mutagenesis (Higuchi *et al*., 1988), and the mutant promoter DNA was cloned in pGL3 vector. Transfections were carried out in control C2C12 and mtDNA-depleted cells using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) by the manufacturer's suggested protocol. Promoter (1 μg) and 0.1 μg of renila luciferase construct as an internal control were used in each transfection. The luciferase activity was assayed using Dual-Luciferase reporter assay system from Promega. Co-transfection with various cDNA constructs was carried out using 0.2 μg of expression construct.

DNA–protein binding by gel mobility shift

Nuclear extracts from control, depleted and reverted C2C12 myocytes were prepared as described (Dignam *et al*., 1983). DNA–protein binding was assayed by using 0.1 to 0.2 ng (20 000 c.p.m.) of ^{32}P-end-labeled double-stranded DNA probes essentially as described (Singh *et al*., 1986). The following synthetic double-stranded DNAs were used either as probes or competitors: SP1 (5′-ATTCGATCGGGGCGGGGCGAA-GC-3′), Egr-1 (5′-GACGGGGCGGGGGCGGGCCCTGT-3′) and Egr-1-MUT (5′-GACGGGGCTAGGGCTAGCCCTGT-3′). Binding reactions (20 ml final volumes) were run at room temperature for 25 min using 1–3 ml of nuclear extracts (7.5 μg of protein in each case) and 1 mg of poly (dI–dC) to minimize non-specific protein binding. The DNA bound complexes were resolved by electrophoresis through 4% polyacrylamide gels using 0.25× TBE (1× TBE = 89 mM Tris base, 89 mM boric acid, 2 mM EDTA) as the running buffer. Competition with 30 molar excess unlabeled DNA and antibody supershift with 1–2 μg of either pre-immune IgG, Sp-1 (Santa Cruz Biotechnology) or Egr-1 (Geneka Biotechnology) antibodies were carried out under standard conditions.

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