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Mitochondrial DNA might be influenced in calprotectin-induced cell death

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ABSTRACT

It is generally believed that calprotectin acts via exclusion of extracellular zinc, and/or binding of calprotectin to a cell membrane receptor, and consequently, activation of a signaling pathway for apoptosis. Recently, we suggested that calprotectin may have an "internal target" within cells. Here, using target theory, we provided evidence that this internal target is DNA. Trypan blue (TB) and dimythylthiazol diphenyl tetrazolium bromide (MTT) assays were used to estimate survival of calprotectin-treated cells. TB assay relies on the viability of cell membrane, while MTT assay relies on the functionality of mitochondria. We demonstrated that MTT-based survival values fit to the "single-hit, single-target" model, while TB-based survival values are best matched to the "single-hit, multi-target" model with N=2. Assumption of DNA as the target of calprotectin is fully consistent with the models, since each mitochondrion contains one chromosome and each "cell" is diploid and contains two chromosome sets. To the best of our knowledge this is the first report that suggests mitochondrial DNA is affected during calprotectin-induced cell death. Furthermore, our results explain why toxicity measures (like LD_{50}), when estimated based on TB assay, are sometimes significantly greater than toxicity measures based on MTT assay.

Keywords: S100A8/A9; MRP8/14; Apoptosis; DNA damage; mtDNA; MTT assay; TB assay

INTRODUCTION

Apoptosis is a type of programmed cell death, in which cells die without releasing their contents to the environment. Some characteristics of apoptosis are ruffling and appearance of smooth-surfaced protuberances of the plasma membrane with its preserved integrity, cellular shrinking, cytoplasmic condensation, cytoskeletal disintegration and chromatin clumping and margination (Fietta, 2006). In addition, a distinctive internucleosome DNA cleavage happens during apoptosis (van Cruchten and van den Broeck, 2002).

Calprotectin is a calcium- and zinc-binding protein heterodimer of S100 protein family (Hunter et al., 2002; Yui et al., 1995) which has apoptosis-inducing activity against various tumor cells and normal cells (Yui et al., 1997). While calprotectin (S100A8/A9) has a central role in apoptosis, calgranulin A (S100A8) and cal-

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granulin B (S100A9) have also important biological functions (Schäfer and Heizmann, 1996; Kerkhoff et al., 1998; Gebhardt et al., 2006).

It is generally believed that calprotectin acts via exclusion of extracellular zinc (Hunter et al., 2002), and/or binding to a cell membrane receptor, and consequently, activation of a signaling pathway for apoptosis (Kerkhoff et al., 2001; Ghavami et al., 2004). Recently, Nakatani et al. (2005) demonstrated that calprotectin becomes internalized by cells. We consequently suggested that calprotectin may have an "internal target" within cells (Zali et al., 2007). We provided evidence from target theory (see below) to support the idea of the influence of such internal targets in calprotectin-induced cell death. Herein, we present the hypothesis that the target of calprotectin is probably genomic (including both nuclear and mitochondrial) DNA. To the best of our knowledge, this is the first report that suggests mitochondrial DNA is affected during calprotectin-induced cell death in a human gastric adenocarcinoma cell line.

THEORY AND ANALYSIS

Target theory was first developed to explain radiation-induced cell death, but it was later found to be useful in the analysis of cell death induced by chemotherapeutics (Valeriote and van Putten, 1975; Gardner, 2000). Based on "single-hit, single-target" (SHST) theory, if there is a single target in each cell and a chemical at a dose D "hits" it, then viability or survival (S) of cells can be estimated by the following equation (Lea, 1955):

$$S = \exp(-qD) \qquad \text{Equation (1)}$$

or alternatively,

$$\ln(S) = -qD \qquad \text{Equation (2)}$$

in which q is the fitting parameter. SHST model is useful in predicting the survival of bacteria and haploid eukaryotic cells, which have a single chromosomal set (Alpen, 1990).

Based on "single-hit, multi-target" (SHMT) theory, if there are N targets in a cell and a chemical at a dose D hits them, then survival (S) of cells can be estimated by the following equation (Lea, 1955):

$$S = 1 - (1 - \exp(-qD))^N \qquad \text{Equation (3)}$$

in which q is the fitting parameter. This model can successfully predict the survival of different cell types (Alpen, 1990). Especially, when N=1, this model reduces to the SHST theory. N=2 for diploid cells, which possess two sets of chromosomal DNA.

We measured relative cellular and mitochondrial survival using TB (trypan blue) and MTT (dimythylthiazol diphenyl tetrazolium bromide) assays, respectively. The principle of the TB assay is that viable cells exclude the dye whereas dead cells take it up. Measurement of survival is achieved by direct counting of viable and dead cells. When calprotectin concentration is C, survival (S_C) can be calculated as:

$$S_C = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \qquad \text{Equation (4)}$$

Since some cells may die even when calprotectin concentration is zero, we calculated "normalized survival" (NS_C) to correct for the natural random cell death:

$$NS_C = \frac{S_C}{S_{zero}} \qquad \text{Equation (5)}$$

In MTT assay, active mitochondrial dehydrogenases convert soluble yellow MTT to insoluble purple formazan. The formazan is then dissolved in a solution containing isopropanol. Afterward, optical density is read against blank reagent with an ELISA reader. Therefore, this method estimates only the survival of "mitochondria" in a cell culture sample, not directly the survival of cells (note that these survival values are not necessarily equal). When calprotectin concentration is C, survival (S_C) can be calculated as:

$$S_C = \frac{\text{Absorbance of calprotectin - treated sample}}{\text{Absorbance of a sample without incubation}} \qquad \text{Equation (6)}$$

Again, "normalized survival" should be calculated by Equation 5.

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We incubated C-131 cells (Zali et al., 2007) with different concentrations of calprotectin. Figure 1 shows that the results of MTT assay are best fitted to the SHST model, while SHMT model with N=2 successfully predicts the cell survival as a function of calprotectin dose.

As explained before, MTT assay estimates the survival of "mitochondria", while TB assay approximates "cell" survival. Each mitochondrion has a single chromosome, while each cell has two sets of chromosomes. The results in Figure 1 are consistent with the assumption that chromosomes are the targets of calprotectin action. Therefore, calprotectin induces cell death by influencing nuclear *and* mitochondrial DNA.

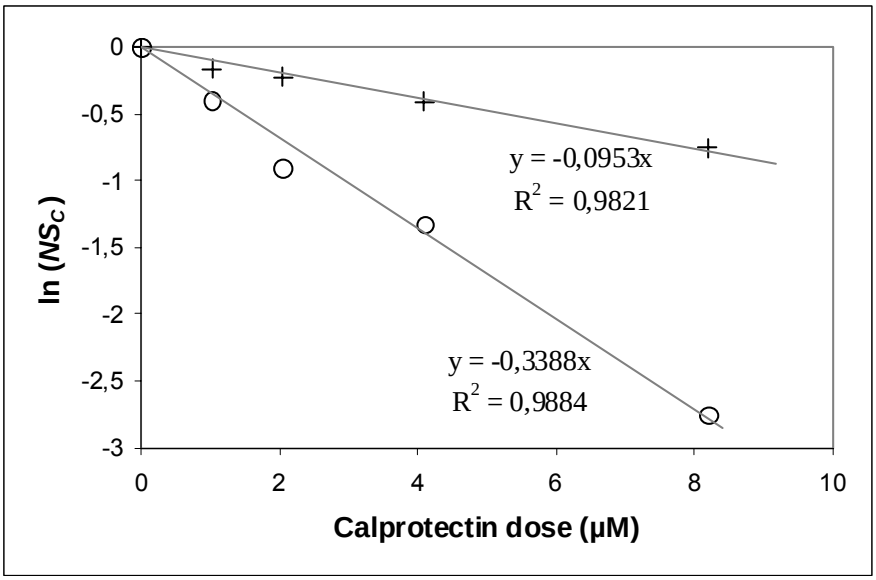

Figure 1A

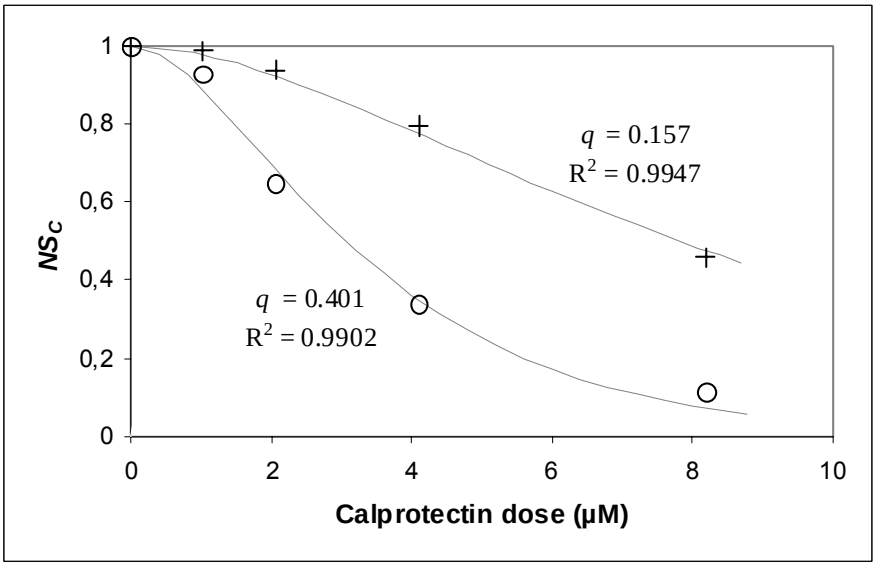

Figure 1B

Figure 1: Normalized survival values for cells incubated with different calprotectin concentrations for 24 (+) or 48 (○) hours. (A) Logarithm of survival values based on MTT assay as a function of calprotectin dose. The fitted line is based on Equation (2). (B) Survival values based on TB assay as a function of calprotectin dose. The experimental data presented in this Figure are similar to the data in Zali et al. (2007); however, the revisited fitted curve is based on Equation (3), that shows the consistency of data with SHMT model with N=2.

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It is well known that nuclear DNA is influenced during apoptosis (van Cruchten and van den Broeck, 2002; Fietta, 2006), but mitochondrial DNA alterations were not reported to normally occur during apoptosis (E. Gottlieb and J. Yuan, personal communications), although there are reports on the mitochondrial DNA damage in apoptosis, specially in neurons (Ozawa et al., 1997; de la Monte et al., 2000). This study is the first report that suggests mitochondrial DNA might also be influenced during calprotectin-induced cell death in a human gastric adenocarcinoma cell line.

As control experiments, we performed similar experiments with etoposide, a well-known apoptosis-inducing drug (Hande, 1998), which binds to topoisomerase II. However, cell survival followed neither SHST nor SHMT models (data not shown). This clearly shows that the patterns of cell death reported in this work depend strictly on calprotectin as the cytotoxic agent and are not generally observed for all apoptosis-inducing agents.

It has been suggested that exclusion of zinc from the extracellular environment is the main mechanism of calprotectin action (Hunter et al., 2002), as zinc chelation has putative cytotoxic effects (Ghavami et al., 2005; Hashemi et al., 2007). It was also suggested that calprotectin might bind to its receptor on cell surface (Kerkhoff et al., 2001; Ghavami et al., 2004), although this hypothesis is not generally accepted. Recently, it has been demonstrated that calprotectin is internalized by cells (Nakatani et al., 2005). This observation leads us to present the hypothesis of the existence of an internal target for calprotectin (Zali et al., 2007). Altogether, it seems that different pathways are involved in the mechanism of action of calprotectin.

It has been demonstrated that reactive oxygen species (ROS) are regulating factors in the initiation of apoptosis signaling. In addition, antioxidant agents can prevent the apoptosis induced by calprotectin (Mikami et al., 1998; Ghavami et al., 2004). ROS can affect mitochondrial outer membrane (MOM) and mitochondrial inner membrane (MIM) and influence their integrity. This mitochondrial membrane permeabilization results in the release of several apoptotic proteins to the cytosol (Alirol and Martinou, 2006), including cytochrome *c*, SMAC/DIABLO, AIF, Endo G, and Omi/HtrA2. Ghavami et al. (2004) suggested that the observed apoptotic activity was induced through classical cytochrome *c*-dependent pathway, with the activation of caspase-3 and caspase-9. This is evidently consistent with the assumption that the targets of calprotectin action are MOM and MIM. In contrast, it has been recently demonstrated that calprotectin can result in DNA fragmentation, even when overexpression of anti-apoptotic Bcl-2 abrogates caspase activation (Viemann et al., 2007). This is in contradiction with the previous models for apoptotic mechanism of calprotectin, whereas it is consistent with our hypothesis, as shows that calprotectin influences DNA even when cells do not undergo caspase-dependent apoptosis.

At the end, we want to emphasize that our model is presented based on the mentioned calprotectin dose range (i.e. 8 µM and less). One should check if the cell survival pattern remains the same out of this range.

WHY THE RESULTS OF MTT AND TB ASSAYS ARE SO DIFFERENT?

In fact, the significant difference between toxicity measures calculated by MTT and TB assays has always been puzzling. For example, LD_{50} values (i.e. the lethal dose that reduces 50 % of viability) based on TB assay is greater compared to LD_{50} values based on MTT assay (de Haan et al., 1993). The present study shows that such a difference is originated from the kinetics of mitochondrion vs. cell death: since mitochondrial death obeys SHST model, it happens faster than cellular death, which follows a SHMT model. Thus, the lethal dose for mitochondria is smaller than the lethal dose for the whole cell, or equally, LD_{50} values based on MTT assay are much smaller than LD_{50} values determined by TB assay.

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