

## Citrate Induces Apoptotic Cell Death: A Promising Way to Treat Gastric Carcinoma?

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**Abstract.** Gastric carcinoma is frequent, particularly in China, and therapy is often inefficient. Because cancer cells are partly or mainly dependent on glycolysis to generate adenosine triphosphate ATP (Warburg effect) and/or to produce precursors (of lipid, nucleotides, etc.) for building new cells, any inhibition of glycolysis may slow down the cell proliferation and/or may kill cells. The antitumor effect of citrate, an anti-glycolytic agent inhibiting phosphofructokinase (PFK) was tested on two human gastric carcinoma cell lines. **Materials and Methods:** Cell viability and morphology were assessed after 24-72 h exposure to citrate (5, 10, 220 mM). Apoptosis was assessed by annexin V-FITC/PI staining and Western immunoblotting. **Results:** A 3-day continuous exposure to citrate led to near destruction of the cell population in both cell lines, apoptotic cell death occurred through the mitochondrial pathway in a dose- and time-dependent manner, associated with the reduction of the anti-apoptotic Mcl-1 protein in both lines. **Conclusion:** Citrate demonstrates strong cytotoxic activity against two gastric cancer lines, leading to an early diminution of expression of Mcl-1 and to massive apoptotic cell death involving the mitochondrial pathway.

Gastric carcinoma is the fourth most common cancer worldwide and represents one of the most frequent causes of death by cancer (1). Each year, approximately 700,000 people die of such cancer, representing about 10% of all

cancer deaths occurring around the world (2). Its frequency is highest in China, where most cases are diagnosed at mid or advanced stages. Even when surgically treated, the 5-year survival of patients is less than 30% (3). Therefore, it is fundamental to find new treatments.

More than 75 years ago, Otto Warburg reported that most cancer cells exhibit increased glycolysis, leading to the secretion of lactic acid, even in the presence of oxygen (4), even considering this to be at the origin of cancer (5). The mechanisms involved in the Warburg effect are currently more and more studied (6-9), while PET scanning, used to detect metastases of solid carcinomas, is a direct application of the glucose avidity of cancer cells (10). Glucose uptake by cancer cells can increase by up to 10 to 15-fold in comparison to normal cells, in relation to the increase of activity and expression of glucose membrane carriers and of most glycolytic enzymes related to the modified metabolism of these cells (6-13). Glycolytic tumors are often considered to be the most aggressive (10, 14) and a decrease of glucose metabolism, visualized by PET, is generally considered a good predictor of the response to cancer therapy (8). For many years, our group has explored the potential benefit of anti-glycolytic agents on tumor growth either *in vitro* or *in vivo*, considering that exploitation of the Warburg effect could represent a novel and promising approach to overcome the frequent resistance of carcinomas, in particular of mesothelioma, to conventional radio- and chemotherapy (15-17). The biochemical and molecular mechanisms leading to the Warburg effect are complex (6-9), but any inhibition of glycolysis may slow down the proliferation of cancer and/or may kill cells, as demonstrated by several studies, including our own (14-20). This appears particularly to be true when glycolysis is the main source of adenosine triphosphate (ATP) for cells, as in clones where mitochondrial ATP supply is compromised (16, 21). Even when mitochondrial function is not impaired (as

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seems to be the case in most cancer cells) (8, 22, 23), any blockage of glycolysis may induce lower production of the metabolic precursors needed for cell proliferation (acetyl-CoA, glycerol, and nicotinamide adenine dinucleotide phosphate (NADPH, H<sup>+</sup>) for fatty acid synthesis; ribose and NAD<sup>+</sup> for nucleotide synthesis; non-essential amino acids for protein synthesis, *etc.*) and, therefore, may at least result in a slowing of cell proliferation.

Apoptosis plays a critical role in the response to current chemotherapy drugs (24) and any glucose deprivation may also lead to apoptosis (15-21). The link between glycolysis and apoptosis is at the hexokinase II (HK II) level (18, 25, 26), the enzyme that converts glucose to glucose-6-phosphate (G6P). HK II maintains voltage anion channel (VDAC) (a component of the mitochondrial permeability transition pore) in the open state, thus counteracting outer mitochondrial membrane permeabilization. Any inhibition of HK II allows removal of HK from this complex, leading to permeabilisation of the mitochondria, release of cytochrome *c* and subsequent caspase activation and apoptosis (18, 25, 26).

Among the various anti-glycolytic agents we have tested, citrate, a strong physiological inhibitor of phosphofructokinase (PFK), appeared to be the most interesting one for theoretical reasons presented in the discussion, and because it gave impressive results on chemoresistant mesothelioma cells when associated with cisplatin (17).

The exposure of two human gastric carcinoma cell lines, BGC-823 and SGC-7901 cells, to citrate was investigated.

## Materials and Methods

**Cell line and culture.** Human gastric cell lines BGC-823 and SGC-7901 were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. These cell lines grow in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cells were maintained in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. Cell viability was evaluated using an inverted microscope by the trypan blue exclusion method at various times after exposure to sodium citrate (5, 10 and 20 mM). All the experiments were performed in duplicate.

**Nuclear morphology study.** After treatment, detached cells were separately collected and adherent cells were dissociated by trypsin/EDTA. The cells were then pooled and collected on a polylysine-coated glass slide by cytocentrifugation, fixed in ethanol/chloroform/acetic acid solution (6:3:1) and incubated for 15 min at room temperature with a 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) aqueous solution. The slides were thereafter extensively washed in distilled water, mounted in Mowiol (Calbiochem, Darmstadt, Germany) and analysed under a fluorescence microscope.

**Annexin V-FITC/PI staining.** Following the incubation, 2×10<sup>5</sup> cells were labeled with 5 µl annexin V-FITC and 2.5 µl propidium iodide (PI) in 100 µl binding buffer for 15 min on ice in the dark to differentiate apoptotic and necrotic cell death using an annexin V-FITC/PI-staining kit (Immunotech, Krefeld, Germany). Afterwards, 150 µl binding buffer was added, and the cell samples were

analyzed immediately using a FACSCalibur flow cytometer and CellQuestPro software (Becton Dickinson, San Jose, USA). Apoptosis was determined when the cells were annexin V-FITC-positive and necrosis when the cells were double positive (annexin V-FITC/PI-positive).

**Western immunoblotting.** After 24 h exposure to citrate (tribasic sodium citrate, pH 7.5; Sigma Aldrich, Saint Quentin-Fallavier, France), the cells were rinsed with ice cold PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris HCl pH 8, 1% Triton ×100, 4 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM aprotinin, 5 mM EDTA, 10 mM NaF, 10 mM NaPPi, 1 mM Na<sub>3</sub>VO<sub>4</sub>) for 30 min on ice. The lysates were clarified by centrifugation at 10,000 rpm for 10 min at 4°C and the protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, USA). Equal quantities of total cellular protein (20 µg) were resolved in a bis-tris-HCl buffered (pH 6.4) 4-12% polyacrylamide gel (NuPAGE® Novex® 4-12% bis-tris gel, Invitrogen, Shanghai, USA) for 40 min at 200 V and electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF) (GE Healthcare, Orsay, France) for 1 h 15 min at 30 V. The membrane was blocked for 1 hour at room temperature in T-TBS (132 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.05% Tween 20) supplemented with 5% non-fat dry milk. The membrane was incubated for 1 h at room temperature in T-TBS-milk with the following primary antibodies: anti-PARP (polyADP-ribose polymerase), anti-caspase 9 and its cleaved form, anti-caspase-3 and anti-cleaved caspase-3 (each 1:1000; Cell Signalling Technology, Beverly, MA, USA), anti-MCL-1 (1:750, and anti-P53 (1:200) (Santa-Cruz Biotechnology, Santa-Cruz, CA, USA) and anti-α-tubulin (1:4000, Sigma, Saint Louis, MO, USA).

After three washes with T-TBS, the membrane was incubated for 1 h at room temperature in T-TBS-milk with adequate peroxidase conjugated secondary antibody (anti-mouse or anti-rabbit IgG; Amersham). After 3 washes with T-TBS and one with TBS, the immunoreactivity was detected by enhanced chemiluminescence using an ECL kit (GE Healthcare).

## Results

Cell growth and viability studies showed that exposure to citrate was highly cytotoxic. Indeed, in both cell lines, citrate induced obvious cytostaticity after 24 h, leading to cytotoxicity being clearly demonstrated after 48 h. At this time, exposure to 10 mM citrate led to a nearly complete disappearance of cancer cells, and after 72 h, no cells remained viable whatever the concentration used (Figure 1).

On inverted microscopic examination, the cells exposed to citrate demonstrated very noticeable cellular detachment that contrasted with the high cell density observed in the non-treated cells. Nuclear staining with DAPI indicated nuclear condensation and fragmentation in the treated cells, strongly suggesting apoptosis, whereas no obvious change was observed in the untreated cells (Figure 2A and 2B). Flow cytometric analysis after double staining with PI and annexin V-FITC showed that apoptosis and necrosis occurred in both cell lines in a dose- and time-dependent manner, whereas no significant cell death was observed in the untreated cells (Figure 2A and 2B).

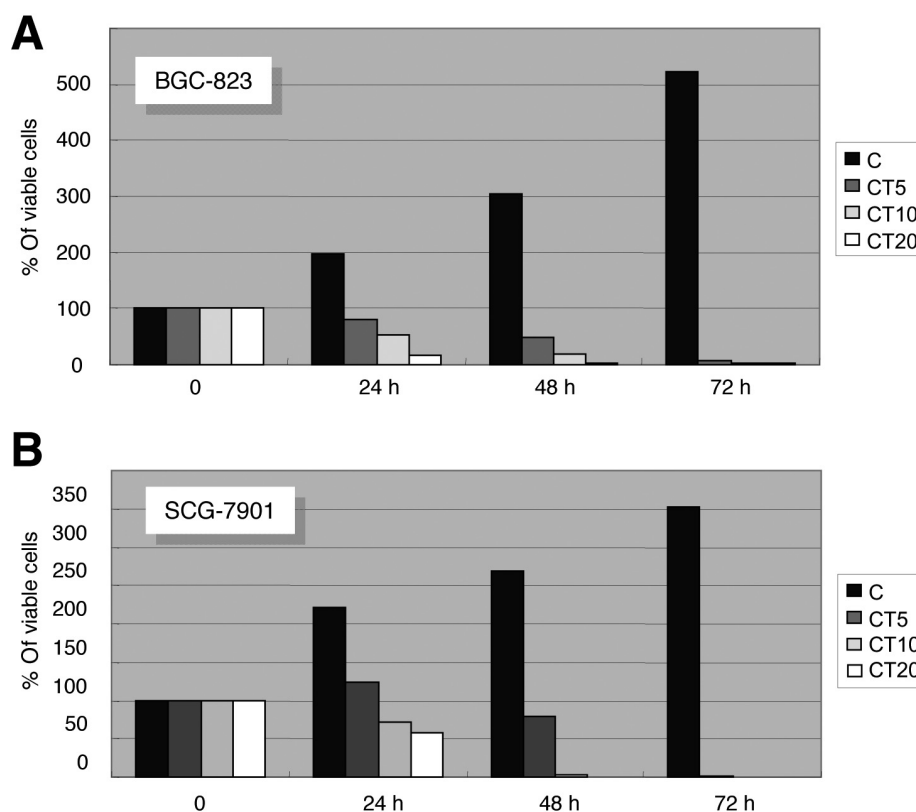


Figure 1. Effect of citrate on cell growth of human gastric carcinoma cell lines BGC-823 (A) and SGC-7901 (B). Kinetic evolution (24-72 h) of cell viability (assessed by trypan blue exclusion test) in response to continuous exposure to citrate (CT) (5, 10, 20 mM).

As demonstrated in Figure 3, Western blot analysis revealed cleavage of caspase-3 and PARP, demonstrating that apoptosis occurred during the first 24 h. Moreover, the cleaved form of caspase-9, was also observed indicating that the mitochondrial pathway was clearly involved in apoptosis. A clear diminution of the expression of the anti-apoptotic protein MCL-1 was also observed in both lines.

## Discussion

Exposure of both human gastric cancer cell lines to 5-20 mM citrate led to massive apoptotic cell death through the mitochondrial apoptotic pathway (activation of caspase-9), in a dose- and time dependent manner. Almost all the cells were destroyed 72 h after exposure to 10 mM of citrate. These results confirmed the anticancer action of citrate that we previously observed in a human mesothelioma MSTO-211H cell line (17), although these cells were less sensitive to citrate than the gastric cells used in this study. In the previous study, citrate sensitized the cells to cisplatin, a drug which was poorly efficient by itself on such cells, leading to

complete cell death through the apoptotic mitochondrial caspase pathway (17). We hypothesized that the depletion of ATP generated by citrate exposure blocked and/or reduced the capacity of the cells to restore cellular and DNA damage secondary to cisplatin, a process necessitating many NAD<sup>+</sup> and ATP molecules, particularly to sustain the activity of PARP in DNA repair (9).

Extrinsic and intrinsic pathways are the two main apoptosis pathways. The extrinsic pathway operates *via* death receptors on the cell surface and the intrinsic pathway, depending on the mitochondria, is activated by loss of growth factor signals or in response to lethal stimuli from inside the cell, such as DNA damages (24). New therapeutic opportunities in cancer are based on the activation of these pathways (27, 28), including the activation of pro-apoptotic receptors, the restoration of p53 activity, the inhibition of the BCL-2-like proteins (BH<sub>3</sub>-mimetics) and of inhibitor of apoptosis proteins (27, 28).

Mitochondrial integrity is regulated by pro- and anti-apoptotic members of the BCL-2 family (27-30). The anti-apoptotic proteins (BCL-x<sub>L</sub>, BCL-2, MCL-1, *etc.*) stabilize

the mitochondrial outer membrane and prevent the release of cytochrome *c* and other apoptotic factors by interacting with pro-apoptotic members of the BCL-2 protein family, such as BAX and BAK (27-30). This sequestration is considered as a major component of resistance to current chemotherapy and has stimulated intensive research to find anticancer agents that promote the release of pro-apoptotic proteins from their anti-apoptotic counterpart to restore apoptosis. Among anti-apoptotic proteins, MCL-1 and BCL-x<sub>L</sub> are overexpressed in many carcinomas and are suspected to play a key role in carcinogenesis and chemoresistance (27-30), particularly in mesothelioma cells as we described (31).

In the current study, citrate reduced MCL-1 expression in both the gastric cancer lines in a dose-dependent manner, in agreement with previous observations in mesothelioma cells (17). Knowing that the concomitant inhibition of MCL-1 and BCL x<sub>L</sub> is sufficient to induce apoptotic death in such cells (31), citrate might have inhibited BCL-x<sub>L</sub> through the activation of BAD, secondary to the inhibition of HK II resulting in the inhibition of PFK. This event, in cooperation with the down-regulation of MCL-1, could thus lead to apoptotic cell death through the mitochondrial pathway.

The mechanism leading to the reduction of MCL-1 protein expression by citrate remains to be elucidated. MCL-1 is subject to rapid turn over (32) and the control of its expression could involve both transcriptional and post-translational mechanisms (28). The interaction of MCL-1 with pro-apoptotic BH<sub>3</sub> only members of the BCL-2 family or with the multidomain Bak is a determinant of its behaviour. Association with some of these partners would lead to stabilization (BAK, PUMA, BIM, NBK/BIK) (29, 33), whereas with others would induce its degradation (NOXA) (34). MCL-1 degradation can involve proteasomes, but can also be a consequence of caspase activity (35). MCL-1 disappearance could thus be either linked to the expression of BH<sub>3</sub>-only proteins in response to citrate exposure (these proteins are indeed considered as stress sensors in the cells) or involve transcriptional mechanisms. This anti-MCL-1 action of citrate is of interest because very few molecules are currently candidates to inhibit MCL-1 expression (28), although its inhibition constitutes a major challenge for the success of many anticancer therapies.

At least five biochemical theoretical considerations (summarized in Figure 4) have led us to test citrate as an anti-energetic agent for treating cancer. Firstly, citrate is a strong inhibitor of glycolysis (11, 12) by blocking PFK and when citrate is abundant, glycolysis is nearly switched off by this regulation (12). At the same time, citrate activates neoglucogenesis by enhancing fructose 1,6-bisphosphatase activity (11, 12). Secondly, citrate is a precursor and a booster of fatty acid synthesis (11, 12). When citrate is abundant in cells, this usually means that energy production (ATP) is sufficient, so oxidative phosphorylation (OXPHOS)

and the Krebs cycle are slowed down or stopped. Citrate moves outside the mitochondrial matrix to the cytosol, where it is converted by ATP-citrate lyase (ACL) to acetyl-coenzyme A (acetyl-coA) and oxaloacetate (OAA). Acetyl-coA serves as a precursor of fatty acid synthesis and is first transformed by acetyl-coA carboxylase (ACC) to malonyl-coA, a reaction requiring ATP. ACC, which is often overexpressed in cancer cells (36, 37), is the key enzyme of fatty acid synthesis regulating the first step of this synthesis (11, 12). It is activated physiologically by citrate (11, 12). It is important to observe that whereas citrate stimulates fatty acid synthesis, which consumes many molecules of NADPH and ATP, it concomitantly inhibits  $\beta$ -oxidation (which would produce much ATP), at least in an indirect manner. Indeed, the first product of the ACC reaction, *i.e.* malonyl-coA, is an inhibitor of acylcarnitine transferase I, which transfers fatty acids from the cytosol to the matrix (11, 12). Interestingly, inhibition of acylcarnitine transferase I also induces apoptosis (38). Thirdly, besides the well-recognized regulative actions of citrate on PFK, fructose 1,6-bisphosphatase and ACC, citrate might also have more hypothetical actions, either on glycolysis or on the Krebs cycle. It may inhibit HK, at least indirectly, by the physiological retroaction of glucose-6-phosphate (G6P) on HK. Indeed, when PFK is blocked by citrate, G6P, which cannot enter the pentose phosphate pathway (PPP) (11, 12) due to citrate-induced ATP depletion, accumulates upstream of PFK, and therefore inhibits HK. Inhibition of HKII in cancer cells may promote apoptosis, because HKII is linked to the MTP and VDAC (18, 25, 26). Fourthly, citrate may inhibit pyruvate dehydrogenase (PDH) (39), the enzyme of the Krebs cycle which links glycolysis and the tricarboxylic cycle, producing acetyl-CoA from cytosolic pyruvate (the end product of aerobic glycolysis). Fifthly, citrate may also inhibit succinate dehydrogenase (SDH) (40), the sole enzyme of the Krebs cycle located at the inner membrane, which couples the Krebs cycle and OXPHOS, because it is a functional member of complex II in the electron transporter chain (ETC).

By blocking ATP production (glycolysis and at least partially the Krebs cycle and  $\beta$ -oxydation), while at the same time stimulating ATP requirement (neoglucogenesis and fatty acid synthesis), citrate leads to a depletion of ATP inside the cell. By diminishing ATP synthesis, and by inhibiting NADPH, H<sup>+</sup> and NAD<sup>+</sup> reforming cycles, citrate inhibits cell proliferation. ATP depletion would lead to apoptosis and/or necrosis in relation to the intensity of the depletion and of the capacity of cells to adapt (41-42).

Indeed, in mesothelioma cells, citrate induced cell death either by an apoptotic mechanism, for MSTO-211H cells (17), or by a poisoning-necrosis mechanism, for NCI-H28 cells (unpublished data). It should be expected that when ATP depletion secondary to citrate exposure is sufficient, it would be more deleterious in cells lacking functional respiration, such as NCI-H28 cells (16),



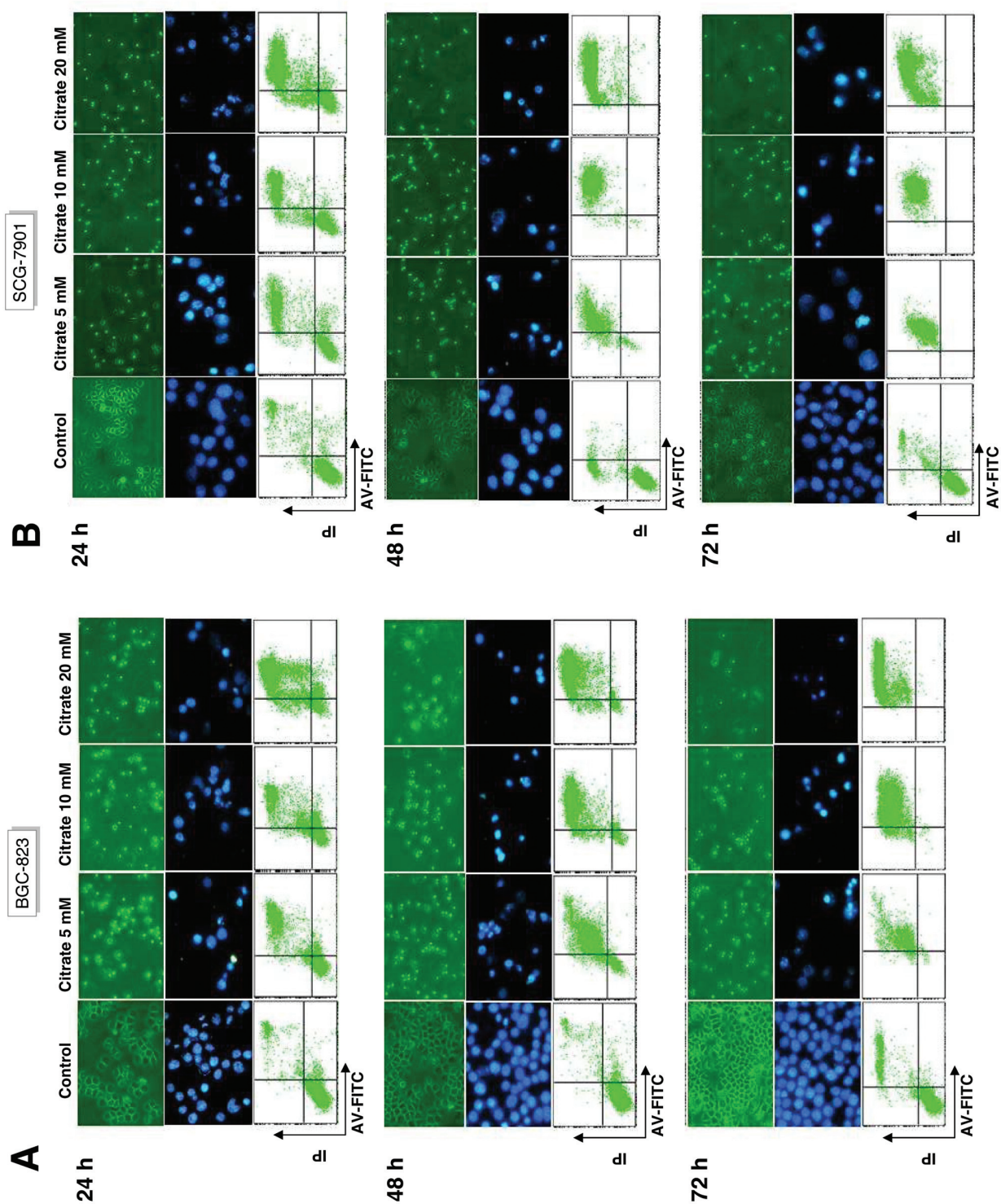


Figure 2. Effect of citrate on cellular and nuclear morphology and on apoptotic/necrotic cell death in human gastric carcinoma cell lines BGC-823 (A) and SGC-7901 (B). Cellular morphology (upper panel), nuclear morphology (centre panel) and annexin V/PI staining (lower panel) were assessed after continuous exposure of cells to citrate.

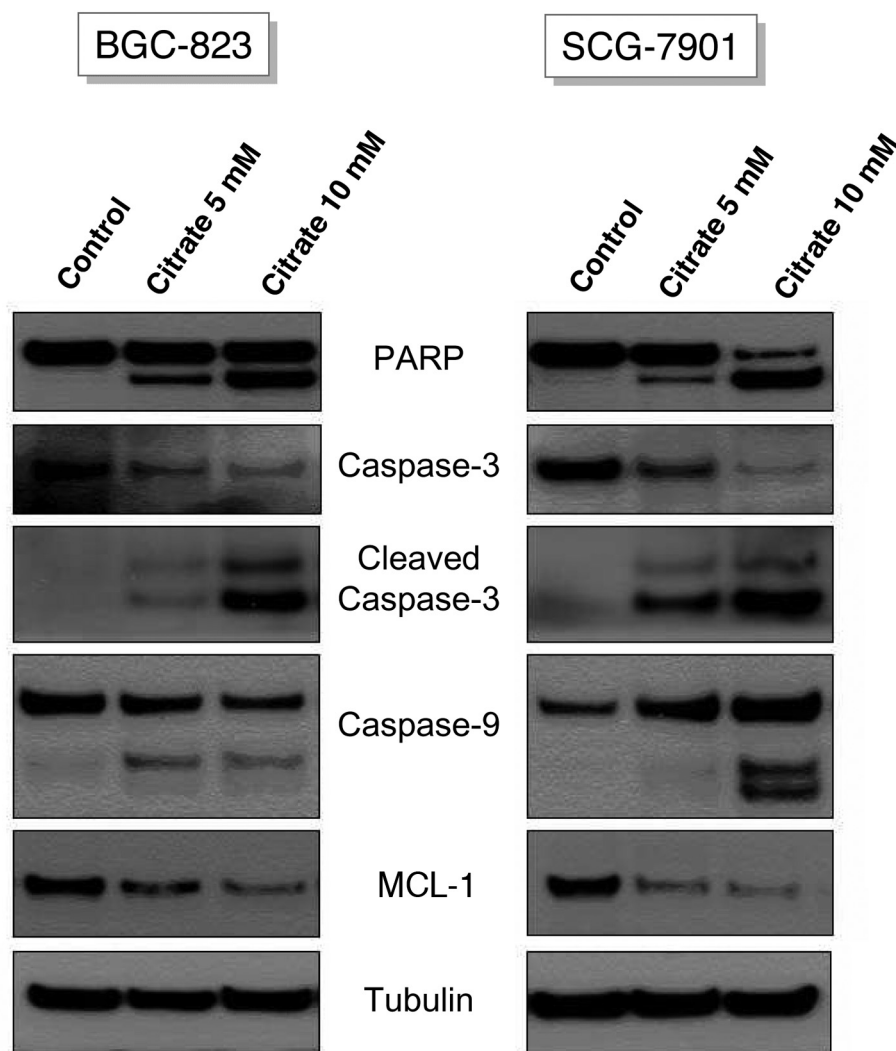


Figure 3. Effect of citrate on mitochondrial apoptotic pathway activation in human gastric carcinoma cell lines BGC-823 and SGC-7901. Caspase-9, caspase-3 and PARP cleavage, as well as MCL-1, expression detected by Western blot after 24 h exposure to citrate. Tubulin was used as loading control.

leading to necrotic death. In the current study, citrate led to early apoptotic death, within 24 h of exposure, as demonstrated by caspase-3 and 9 activation. In contrast, cytometric analysis performed after 24 h showed mainly necrotic death features that could correspond to post-apoptotic necrosis, as frequently observed *in vitro* because apoptotic cells are not removed by macrophages or neighbouring cells.

Citrate could have also non-energetic anticancer actions, as well as reduction of the anti-apoptotic protein MCL-1, citrate could also promote the formation of reactive oxygen species (ROS) since a sudden elevation of citrate concentration inside the cell might immediately stimulate the Krebs cycle. This could happen especially when cells present some alterations of their respiratory chain complex resulting in dysfunctional

OXPHOS, and when their capacity to reduce ROS is exceeded (7, 43). Because the anticancer effect of multiple conventional treatments (*e.g.*, ionizing radiation, etoposide, arsenates, *etc.*) is based on their ability to stimulate ROS production, leading to apoptotic death of cells, this potential action of citrate should be kept in mind, even though it may seem contradictory to previous hypothesis of inhibition of the Krebs cycle by citrate. It should be noted that the redox system requires a great quantity of NADPH for its reducing enzymes (such as glutathion reductase) (7, 12, 27, 43). Because citrate might inhibit PPP from producing NADPH,  $H^+$  (requiring ATP), while enhancing fatty acid synthesis (consuming much NADPH,  $H^+$ ), citrate might finally reduce the pool of NADPH,  $H^+$  and therefore reduce the activity of the redox system.





energy, without adverse effect on normal cells, which are mainly in a steady quiescent state and therefore less sensitive to energy inhibition.

To our knowledge, the toxicity of high citrate doses remains unknown. One author has reported no severe side-effects (gastric acidity and the risk of hypocalcaemia must be oral sodium citrate prevented) and after a daily oral dose of 0.27 g per kg during several months, observed a 50% decrease in calcitonin level in a patient suffering from a medullary thyroid carcinoma (46, 47).

Finally, for all these theoretical and practical reasons, it would be interesting to test citrate, primarily in association with chemotherapy such as cisplatin (17). In gastric carcinomas, it would be interesting to administer citrate orally for direct contact with tumors at early stages, or to treat advanced stage, peritoneal carcinomatosis by administering citrate in the peritoneum, in association with cisplatin. Toxicity studies are currently being performed to evaluate possible adverse side-effects of citrate *in vivo*, as well as the effect of various citrate administration protocols on the growth of various tumor cells.

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