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Ascorbate on Cell Growth and Differentiation

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Ascorbate, an essential nutrient in humans, primates, and guinea pig, is involved in many cellular functions. Ascorbate also modulates cell growth and differentiation. Ascorbate can reduce or stimulate the growth of tumor cells, depending on the cell type. The inhibitory effect is not specific for the biological active isomer L-ascorbate, and isoascorbate and D-ascorbate are more effective in reducing cell growth than L-ascorbate. These results indicate that ascorbate has a cytotoxic effect by killing cells directly, rather a cytostatic one. However, only L-ascorbate is able to stimulate cell growth, but the mechanism of this stimulation is still unknown. L-Ascorbate stimulates the *in vitro* differentiation of several mesenchyme-derived cell types by altering the expression of multiple genes as the cell progresses through specific differentiation programs. Stimulation of collagen matrix at gene transcription, mRNA stabilization, hydroxylation, and secretion is a key role for L-ascorbate. L-Ascorbate also prevents cell transformation by stabilization of the differentiated state and cooperates with other agents to induce differentiation in a leukemia cell line.

KEY WORDS: Vitamin C; tumor growth; mesenchymal differentiation.

INTRODUCTION

Ascorbic acid (ASC) is involved in many physiological functions. It is an essential nutrient in humans, primates, and guinea pig which lack the last enzyme in its synthesis pathway (Chatterjee, 1978). Many of the important functions of ASC occur intracellularly where its function seems to be the provision of electrons to keep prosthetic metal ions (cuprous for monoxygenases and ferrous iron for dioxygenases) in the reduced form, rather than being directly involved in the catalytic cycles (Padh, 1990). Among the extracellular functions, ASC can act as a scavenger of reactive oxygen species, thus completely protecting the lipid against detectable peroxidative damage (Frei *et al.*, 1989).

Antioxidant micronutrients appear to play important roles in protecting the body against cancer. ASC may reduce cancer risk by inhibiting the formation of N-nitroso compounds in the stomach (Block, 1991). ASC also has a beneficial effect on the immune system and thereby can protect against cancer by enhancing tumor surveillance by the immune system (Byers and Perry, 1992).

ASCORBATE AND TUMOR GROWTH *IN VIVO*

Increased intake of ASC prolongs the survival times of patients with terminal cancer of different kinds (Cameron and Pauling, 1976). Large amounts of ASC in animal laboratory diets also reduce the growth of spontaneous mammary tumor in mice (Pauling *et al.*, 1985), inhibit the growth of solid sarcoma 180, Ehrlich ascites carcinoma, and mammary carcinoma in mice (Gruber *et al.*, 1980; Liotti and Talesa, 1982).

Contrary to those observations, investigators have reported that ASC supplementation did not affect the growth of chemically induced colon cancer (Shirai *et al.*, 1985), bladder, (Soloway *et al.*, 1975), and mammary cancer (Abul-Hajj and Kelliher, 1982). Moreover, results of some studies indicated that the

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ASC effect on tumor growth is dose-dependent. There are also reports of inhibition in tumor growth when ASC was supplemented at high doses, but at low doses ASC showed a stimulatory effect on growth (Pauling *et al.*, 1985; Dunham *et al.*, 1982; Liotti *et al.*, 1984). Growth of chemically induced tumors in guinea pigs was unaffected by high doses of ASC while tumor regression was observed at low doses (Migliozzi, 1977). Also it has been reported that ASC is required for tumor growth (Brunschiwg, 1943; Migliozzi, 1977) and that ASC levels are higher inside the tumor than in the surrounding tissues (Hanck, 1985). In human cancer, it has also been statistically demonstrated that high doses of ASC prolong the survival time of patients with terminal cancer (Cameron and Pauling, 1976), but, on the other hand, it may produce no benefits (Moertel *et al.*, 1985).

To increase the controversy over the effect of ASC on tumor growth, it has been reported that ASC can act as a tumor promoter. L-Ascorbic acid is an amplifier of tumor promotion in rat bladder carcinogenesis in combination with sodium salts (Fukushima *et al.*, 1988a). Neither ascorbic acid nor sodium chloride were found to be tumor promoters (Fukushima *et al.*, 1988b). Although the mechanisms that promote urinary bladder epithelial proliferation in response to sodium ascorbate remain to be clarified, a correlation has been reported between this tumor-promoting activity and the hyperpolarization of the epithelial cells (Asamoto *et al.*, 1992). Administration of 5% sodium ascorbate in the diet promoted bladder carcinogenesis, but similar amounts of ascorbic acid did not (Shibata *et al.*, 1992). These authors also reported that ascorbic acid enhanced epithelial hyperplasia and DNA synthesis in chemically induced forestomach tumors (Shibata *et al.*, 1992) probably by increasing the glutathione levels in the tissue.

ASCORBATE AND CELL GROWTH IN CULTURE

Studies *in vitro* have also demonstrated that ASC at concentrations ranging between 0.01 to 0.5 mM killed or suppressed the cell growth of a wide range of cell lines including HEP2 and KB cells (Bishun *et al.*, 1978), ascites cells (Liotti *et al.*, 1984, 1986), bone marrow cells (Park *et al.*, 1980), and melanoma cells (Bram *et al.*, 1980). In general, ASC was more toxic for malignant cells than for normal cells (Leung *et al.*, 1993).

The most sensitive among all tested cell lines were melanoma cells (Bram *et al.*, 1980). The inhibition of cell growth by ASC has been reported to be increased by copper addition in various melanoma and normal cells in culture (Bram *et al.*, 1980; Stich *et al.*, 1976). Melanoma cells contain higher doses of copper than other cell types (Bram *et al.*, 1980), and the stimulation of melanogenesis in these can be induced *in vitro* by $CuSO_4$ addition (De Pauw-Gillet *et al.*, 1987). The presence of copper ions in solution catalyzes the oxidation of ASC, forming toxic products such as hydrogen peroxide and many other ASC derivatives such as γ-crotonolactone, 2-furfural, 3-hydroxy-2-pyrone, 5-methyl-3,4-dihydroxytetrone, etc. (Tsao *et al.*, 1992).

Hydrogen peroxide is toxic for cells lacking the protective enzyme catalase. In fact, malignant cells have low levels of catalase activity, and this property has been used to explain the toxic effect of ASC on these cells. When catalase is supplied to ascites cells in culture, ASC failed to inhibit cell growth (Liotti *et al.*, 1986).

Other chemicals derived from ASC oxidation also showed an inhibitory effect on the growth of human mammary tumor xenografts (Tsao *et al.*, 1992). These results could explain previous studies reporting that ASC only inhibited tumor growth when it was supplemented in drinking water but not if it was administered in the dry diet, or when freshly prepared ASC solutions was administered directly into the throat of the animal (Liotti and Talesa, 1982; Tsao *et al.*, 1977), suggesting that the inhibiting effect of ASC was produced by its degradation products formed in solution (Tsao *et al.*, 1992). Recently this hypothesis has been also demonstrated *in vitro* by Leung *et al.* (1993). Two isomers of ASC, isoascorbate and D-ascorbate, were as effective as ASC in reducing cell growth. As isomers have only 5% of the antiscurvy potency, the effect was due to its chemical properties and was not related to the metabolic effect at cellular levels. These results could indicate that ASC has a cytotoxic effect by killing cells directly rather than a cytostatic one.

However, some studies have reported a stimulatory effect of ASC on cell growth for different cell lines in culture. Hata's group observed a 40% stimulation in DNA synthesis when cultures of human skin fibroblasts were supplemented with 0.1 mM ASC daily for 5 days (Hata *et al.*, 1988). These cells were also stimulated for three weeks by an ASC derivative, L-ascorbic acid 2-phosphate, which is stable in aqueous solution. Similar effects of this compound

were observed in all cell lines tested, such as dermal fibroblasts, embryonic lung fibroblasts, rat skin fibroblasts, and smooth muscle cells (Hata and Senoo, 1989). Also Navarro *et al.* (1992) have reported ASC potentiation of serum-induced S-phase entry of quiescent BALB/c 3T3 cells. Addition of 0.2 mM ASC stimulated DNA synthesis induced by different amounts of fetal calf serum between 40 to 60%.

It has also been reported that ASC modulates the growth of leukemia cells *in vitro*. The first evidence was the finding that ASC was an essential requirement for the colony growth of mouse plasmacytoma cells *in vitro* (Park *et al.*, 1971). The effect was specific for the biological active form, L-ASC. Optical isomers D-ASC and isoascorbate showed little effect. ASC precursors or the other major antioxidant present in the plasma, alpha-tocopherol, were ineffective. ASC had opposite effects on human leukemic cells. ASC enhanced the growth of culture derived from some patients but suppressed cell growth in cultures derived from others (Park *et al.*, 1980; 1992; Park and Kimler, 1991). Enhancement or suppression were not random phenomena. Repeat studies showed that growth stimulation or suppression was consistent within each patient. This dual effect has not yet been explained.

It is assumed that ASC plays an essential role in oxidation–reduction reactions. ASC can act as free radical scavengers, but it can also act as prooxidant through its free radical (AFR). AFR stimulates the growth of the human promyelocytic cell line HL-60 in the presence of a limited amount of fetal calf serum (FCS, 1%), but no stimulation was observed in cultures supplemented with 10% FCS (Alcaín *et al.*, 1990). ASC also stimulated, in the same range, the proliferation of HL-60 cells. ASC is unstable in solution and undergoes autoxidation, losing sequentially two electrons, giving first the intermediate AFR and then dehydroascorbate (Winkler, 1987; Iyanagi *et al.*, 1985). However, the fully oxidized form, dehydroascorbate, was ineffective (Alcaín *et al.*, 1990). ASC and AFR produced a general reduction of the HL-60 cycle length without altering cell distribution frequencies through the cycle (González-Quevedo *et al.*, 1991). These cells growing in 1% FCS-supplemented media can be stimulated by the external electron acceptor ferricyanide. Both AFR and ferricyanide are able to extract electrons from the internal NADH through the plasma membrane (Alcaín *et al.*, 1990; Navas and Burón, 1990; Burón *et al.*, 1993). When AFR accepts an electron, it is reduced back to ASC. In fact, ASC was detected in cell culture only when cells were present, and the remaining ASC was also dependent on the plated cell number (Alcaín *et al.*, 1990; Alcaín *et al.*, 1991). It has been proposed that AFR stimulated the cell growth by activation of the transplasma membrane electron transport system (see Navas *et al.*, 1994 for a review).

ASCORBATE AND MESENCHYMAL DIFFERENTIATION

ASC stimulates the *in vitro* differentiation of several mesenchyme-derived cell types such as adipocytes (Taylor and Jones, 1979), myoblasts (Vogel *et al.*, 1987; Horovitz *et al.*, 1989), chondrocytes (Leboy *et al.*, 1989; Gerstenfeld and Landis, 1991), osteoblasts (Aronow *et al.*, 1990; Franceschi and Young, 1990), and odontoblasts (Heimrika-Wagner *et al.*, 1982). Although the function of ASC in mesenchyme-derived connective tissues has not been defined, a common finding in all the reported studies is that ASC can alter the expression of multiple genes as the cell progresses through specific differentiation programs.

Stimulation of the collagen matrix at gene transcription, mRNA stabilization, translation, hydroxylation, or secretion levels are key roles for ASC in mesenchymal differentiation (Schwartz, 1985) besides its biochemical roles, recently reviewed by Padh (1990, 1991). A number of specific inhibitors of collagen synthesis and secretion have been used to show that ASC affects differentiation by stimulating collagen matrix synthesis and deposition rather than by having a more direct action on differentiation markers. When ASC is added to preosteoblast cultures, collagen synthesis increases two to three days before any induction of osteoblast marker mRNA is seen (Franceschi, 1992). Furthermore, collagen synthesis inhibitors are effective only in early stages of differentiation (Habuchi *et al.*, 1985), and similar results have been reported for myotube formation (Nadan *et al.*, 1990).

It has been hypothesized that ASC would be required in the differentiation of mesenchyme-derived tissues, providing a permissive environment for tissue-specific gene and differentiation markers expression (Schwartz *et al.*, 1987; Franceschi, 1992). There are studies showing that osteoblast proliferation could be controlled by ASC through an integrin-mediated

pathway in which integrin-type cell-surface adhesion receptors, which bind collagen and other matrix molecules, would relay information from the extracellular matrix to the cell interior (Albelda and Buck, 1990). Peptides containing a sequence found in the cell-binding domains of several extracellular matrix molecules (such as collagen type I or fibronectin) have been reported to produce a blockage of the proliferative activity induced by ASC (Harada *et al.*, 1991).

The establishment of a mechanism explaining the relationship between the matrix synthesis induced by ASC and the phenotypic differentiation of mesenchyme-derived cells is of crucial interest in the understanding of connective tissue diseases.

ASCORBATE AND CHONDROCYTE DEVELOPMENT

There is a striking parallel between the changes seen during the maturation of the growth plate chondrocytes *in vivo* (elevation of alkaline phosphatase, synthesis of type X collagen, matrix vesicle biogenesis, and mineralization) and the changes induced by exposing cultured chondrocytes to ASC (Saphiro *et al.*, 1991). ASC is required for the synthesis of specific components of the cartilage extracellular matrix, the formation of matrix vesicles, and calcium accumulation (Wu *et al.*, 1989).

Furthermore, maturation of chondrocytes in the growth cartilage is spatially and temporally linked to a series of unique changes in cellular energy metabolism (Kakuta *et al.*, 1986; Saphiro *et al.*, 1988). Analysis of the metabolic data from chondrocytes in culture suggests that ASC maintains the energy status of cells of the growth cartilage by utilization of the mitochondrial oxidative phosphorylation pathway. ASC promotes oxidative metabolism by inhibiting utilization of pyruvate for anaerobic glycolysis and, as a result, substrate (pyruvate) is made available for mitochondrial oxidative reactions (Saphiro *et al.*, 1991). Earlier studies have shown elevation of oxygen uptake by chondrocytes treated with ASC (Ramp and Thornton, 1968).

However, a sustained decrease in mitochondial oxidative metabolism is observed in the hypertrophic zone of the growth plate *in vivo* (Kakuta *et al.*, 1986). This difference can be due to the architecture of the growth plate in the hypertropic region where gradients of oxygen and nutrient availability may exist and down-regulate oxidative energy metabolism. In culture, environmental conditions do not impose such a metabolic stress on cells.

STABILIZATION OF THE DIFFERENTIATED STATE: A ROLE FOR ASCORBATE

Several possible mechanisms for the prevention of oncogenic transformation by ASC compounds have been reported. Dehydroascorbate, as an electron acceptor, has been proposed to have antitumor activity via an antimitotic effect (Edgar, 1970). Also, an antioxidant mechanism of ASC has been proposed for the chemopreventive action on chemical carcinogenesis (Slaga and Bracken, 1977; Liehr and Wheeler, 1983).

Benedict *et al.* (1980; 1982) have shown that ASC, at noncytotoxic concentration, completely prevented the expression of oncogenic transformation in C3H/10 T 1/2 mouse embryo cells even if treatment began as late as 23 days after exposure to the carcinogen methylcholanthrene. Recently, the inhibition of transformation in these cells by ASC has been associated with both regulation of the redox potential and turnover of glycoproteins and lipids (Ibric *et al.*, 1991). These authors reported that ASC at concentrations that did not inhibit transformation did not modify the NAD^+/NADH ratio. However, when ASC was kept continuously in the medium with an inhibitory effect on transformation, this ratio was significantly altered with a 70% increase over control, returning to control levels after removal of ASC. They also showed alterations of matrix collagen, glycoproteins, and lipids during treatment with ASC in a dose-dependent manner.

The multiple effects of ASC on cell metabolism complicate the attempts to elucidate the basis for its inhibition of neoplasic transformation. However, the consideration of transformation phenomena as a cooperative process gives a context for understanding how ASC can influence the stabilization of the differentiated state (Bissel *et al.*, 1980). In primary avian tendon cells, the transition to the transformed phenotype by Rous sarcoma virus depends on the phenotype of the neighboring cells. Within this context, ASC can be effective in protecting cells from damage in a highly oxygenated culture environment or reducing viral particle production and, as a consequence, the transition to the transformed state (Schwarz, 1991).

EFFECT OF ASCORBATE ON HL-60-INDUCED DIFFERENTIATION

HL-60 cells are induced to monocytic differentiation by exposure to a variety of compounds with loss of their proliferative capacity and acquisition of maturation features of macrophage-like cells. Among these inducers are the phorbol ester 12-O-tetradecanoylphorbol (TPA) (Rovera *et al.*, 1979) and the active form of vitamin D3, 1,2,5(OH)2 or calcitriol (Taimi *et al.*, 1991).

Calcitriol is implicated in hematopoietic and immune differentiation at physiological concentrations, and its therapeutic potential for malignancies is under study (Dusso *et al.*, 1991). However, the severe complications of hypercalcemia in patients treated with calcitriol have contributed to current efforts to develop analogs having low calcemic activity but retaining antiproliferative effects in order to obtain a higher benefit/risk ratio (Walters, 1992).

Addition of ascorbate to cell cultures increases the specific differentiation markers of HL-60 cells induced by calcitriol (unpublished data). This effect is proportional to both concentration of ascorbate and time of induction. The best results in obtaining cells with differentiated phenotype are obtained with 10^{-8} M calcitriol in the presence of 0.5 mM ascorbate. Under these conditions ascorbate increased by 10–15% the number of CD11b positive cells with respect to induction by calcitriol itself, within the first 24 h of treatment.

The mechanism responsible for this action of ascorbate on HL-60 cells induced by calcitriol is unknown. The ascorbate free radical is considered an external electron acceptor of plasma membrane redox system. Recently, we have reported that this redox system in HL-60 cells is modulated during TPA-induced differentiation (Burón *et al.*, 1993). TPA produced a transient increase of transplasma membrane redox activity and pyridine nucleotide levels and a shift in the NAD^+/NADH ratio.

In the future, a detailed study of the plasma membrane redox system during HL-60 differentiation induced by calcitriol in the presence of ascorbate would clarify whether this system can account for the effect caused by ASC. On the other hand, the possible influence of ASC on the action mechanisms of calcitriol, including phospholipid metabolism, protein kinase C induction, cAMP levels, or even proto-oncogenic expression, should not be discarded.

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