Human cells transport dehydroascorbic acid through facilitative glucose transporters, in apparent contradiction with evidence indicating that vitamin C is present in human blood only as ascorbic acid. On the other hand, activated host defense cells undergoing the oxidative burst show increased vitamin C accumulation. We analyzed the role of the oxidative burst and the glucose transporters on vitamin C recycling in an in vitro system consisting of activated host-defense cells co-cultured with human cell lines and primary cells. We asked whether human cells can acquire vitamin C by a “bystander effect” by taking up dehydroascorbic acid generated from extracellular ascorbic acid by neighboring cells undergoing the oxidative burst. As activated cells, we used HL-60 neutrophils and normal human neutrophils activated with phorbol 12-myristate 13-acetate. As bystander cells, we used immortalized cell lines and primary cultures of human epithelial and endothelial cells. Activated cells produced superoxide anions that oxidized extracellular ascorbic acid to dehydroascorbic acid. At the same time, there was a marked increase in vitamin C uptake by the bystander cells that was blocked by superoxide dismutase but not by catalase and was inhibited by the glucose transporter inhibitor cytochalasin B. Only ascorbic acid was accumulated intracellularly by the bystander cells. Glucose partially blocked vitamin C uptake by the bystander cells, although it increased superoxide production by the activated cells. We conclude that the local production of superoxide anions by activated cells causes the oxidation of extracellular ascorbic acid to dehydroascorbic acid, which is then transported by neighboring cells through the glucose transporters and immediately reduced to ascorbic acid intracellularly. In addition to causing increased intracellular concentrations of ascorbic acid with likely associated enhanced antioxidant defense mechanisms, the bystander effect may allow the recycling of vitamin C in vivo, which may contribute to the low daily requirements of the vitamin in humans.

Vitamin C is essential to human physiology and, because humans are unable to synthesize the vitamin, there is an absolute dependence on dietary vitamin C (1). Only reduced vitamin, ascorbic acid, is present in human plasma and in cells and tissues, and the cellular content of vitamin C can exceed by several orders of magnitude the plasma levels of the vitamin (2). Human cells transport ascorbic acid in a concentrative manner through sodium-ascorbic acid co-transporters (3–8); they also transport oxidized vitamin C, dehydroascorbic acid, down its concentration gradient through facilitative glucose transporters (9–18). Although all cells express facilitative glucose transporters, the absolute specificity of these transporters for dehydroascorbic acid is in apparent contradiction to evidence indicating that vitamin C is present in human plasma only as ascorbic acid. Human host defense cells, however, have potent oxidative properties that are greatly enhanced by triggering of the oxidative burst, which leads to increased accumulation of vitamin C by the activated cells (15, 19–22). It is believed that the increased vitamin C uptake observed in the activated cells is caused by the oxidation of ascorbic acid to dehydroascorbic acid, which is then rapidly transported intracellularly by the glucose transporters. An important implication of this concept is that the dehydroascorbic acid generated by cells secreting oxidants can be transported intracellularly by any cell present in the immediate area because glucose transporters are present in all cells and tissues. Thus, activated neutrophils participating in inflammatory reactions may provide other cell types with increased antioxidant protection. Using phorbol 12-myristate 13-acetate (PMA)1-activated cells (HL-60 neutrophils and normal human neutrophils), we validated the concept that superoxide produced by activated cells does oxidize extracellular ascorbic acid to dehydroascorbic acid. We used co-culture assays to demonstrate that human cells can acquire vitamin C by a “bystander effect,” taking up dehydroascorbic acid generated by superoxide anions produced by activated neighboring cells and subsequently accumulating ascorbic acid. We propose that the bystander effect may allow the cellular recycling of vitamin C by cells.

EXPERIMENTAL PROCEDURES

The cell lines were maintained in culture at a cell viability greater than 95% as assessed by trypan blue exclusion. The following established cells and cell lines were used: a neutrophilic subline of HL-60 cells (15, 23); normal mammary epithelial cells and the breast cancer cell lines MCF-7 and MDA-468 (24–26); the human melanoma cell line GMEL (27); and the human prostate adenocarcinoma cell line DU-145 (28). Strains of normal human mammary epithelial cells (normal breast cells) were obtained from Clonetics (San Diego, CA) and were cultured using their media and supplements. Fresh normal human neutrophils were isolated by fractionation in Ficoll-Hypaque (15). Human umbilical vein endothelial cells were obtained from umbilical cords by collagenase digestion, cultured in medium 199, and used within the first three passages (29). Discarded umbilical cords from full-term normal pregnancies were donated by the Regional Hospital in Concepción.

1 The abbreviation used is: PMA, phorbol 12-myristate 13-acetate.

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Uptake assays were performed in incubation buffer (15 mM Hepes pH 7.6, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2) containing 0.1 mM EDTA (pH 8.0) at 4°C. The extracts were stored at −70°C. Cells containing vitamin C in solution were adjusted to 60% methanol and 1 mM EDTA and stored at −70°C. HPLC analyses were performed using a Whatman strong anion exchange reverse-phase column (Partisil 10 SAX, 4.6 mm × 25 cm, 10-μm particle) (15). Dehydroascorbic acid eluted at 4.6 min, and ascorbic acid eluted at 9.4 min. The elution of dehydroascorbic acid was monitored by radioactivity, whereas ascorbic acid was monitored by both radioactivity and absorbance at 266 nm.

Production of superoxide was quantitated by measuring the superoxide-dismutase inhibitable reduction of ferricytochrome c. Cells were suspended in incubation buffer containing ferricytochrome c at 1 mg/ml and activated by the addition of PMA. The reduction of ferricytochrome c was monitored by determining the change in absorbance at 550 nm and using an extinction coefficient of 21,000 M−1·cm−1.

RESULTS

Production of Extracellular Dehydroascorbic Acid by Activated HL-60 Neutrophils—Compared with untreated cells, HL-60 neutrophils treated with PMA and incubated with ascorbic acid showed a markedly increased uptake of vitamin C. At 60 min, PMA-treated cells showed a 60-fold increase in vitamin C accumulation (50 pmol/106 cells) compared with control cells (8 pmol/106 cells) (Fig. 1A). Ten μM cytochalasin B, but not cytochalasin E, blocked vitamin C uptake by the activated cells (Fig. 1A). Superoxide dismutase blocked the uptake of vitamin C by the PMA-treated HL-60 neutrophils (Fig. 1A), whereas catalase had no effect (Fig. 1A). Thus, the full effect of PMA on vitamin C uptake by the HL-60 neutrophils can be blocked by a specific inhibitor of the glucose transporters and an enzyme that dismutates superoxide anions.

Consistent with the above observations, PMA-treated cells generated substantial amounts of superoxide anions. At 60 min, PMA-treated cells generated 19 pmol/106 cells compared with less than 0.5 pmol/106 cells in control cells (Fig. 1B). Parallel experiments revealed that neither cytochalasin B nor cytochalasin E, at 10 μM, affected the production of superoxide by the activated cells (Fig. 1B), thereby confirming the specificity of the inhibitory effect on the glucose transporter. Superoxide dismutase blocked the generation of superoxide by the PMA-treated HL-60 neutrophils, whereas catalase showed no effect (Fig. 1B). Thus, the full effect of PMA on vitamin C uptake by the HL-60 neutrophils can be directly linked to enhanced production of superoxide anions. When the HL-60 neutrophils were treated with PMA in buffers in which the NaCl was replaced with choline chloride, the rate of vitamin C uptake increased in a similar manner and approached a similar plateau in the presence or in the absence of sodium ions, and uptake was again specifically inhibited by cytochalasin B (data not shown).

From the above data, we concluded that the PMA-activated HL-60 neutrophils directly transport locally generated dehydroascorbic acid through the glucose transporters. We used HPLC to directly verify the generation of dehydroascorbic acid by the activated HL-60 neutrophils. Treatment of HL-60 neutrophils with PMA resulted in the time-dependent oxidation of extracellular ascorbic acid (Fig. 1C). At 60 min, ~40% of the radioactivity eluted in the position corresponding to ascorbic acid, compared with greater than 95% in control samples treated with superoxide dismutase. These experiments revealed the time-dependent generation of two radioactive peaks, a first one eluting at 4.6 min and corresponding to dehydroascorbic acid, and a second one eluting at 11 min, which corresponded to dehydroascorbic acid hydrolysis products (data not shown). We estimated the total amount of dehydroascorbic acid generated in the presence of PMA-treated cells as the sum of the material contained in the HPLC peaks eluting at 4.6 and 11 min, which revealed that more than 60% of the original...
ascorbic acid present in solution was oxidized to dehydroascorbyc acid in 60 min (Fig. 1C).

Bystander Effect—We tested the validity of the “bystander effect” concept in an in vitro model consisting of activated HL-60 neutrophils co-cultured with the human prostate cancer cells DU-145 (the bystander cells). The prostate cells grow in monolayers and adhere to the culture plates and therefore are easily separated from the HL-60 neutrophils that grow in suspension, permitting the determination of the content of vitamin C in each cell type separately. There was some uptake of vitamin C in untreated prostate cells incubated alone, probably because of the presence of low levels of dehydroascorbic acid in the sample of ascorbic acid, and that uptake was not increased by treatment with PMA (Fig. 2A). On the other hand, DU-145 cells incubated in the presence of activated HL-60 neutrophils showed increased uptake of vitamin C (Fig. 2A). At 60 min, they contained 6 nmol of vitamin C per million cells, an amount 15-fold higher than that accumulated by PMA-treated prostate cells incubated alone. Parallel experiments revealed a similar time-dependent production of superoxide anions (rate: 0.17 nmol/106 HL-60 neutrophils × min) by co-cultures of PMA-treated HL-60 neutrophils and prostate tumor cells compared with cultures of PMA-treated HL-60 neutrophils alone (Fig. 2B). The DU-145 cells did not produce superoxide anions when cultured under similar conditions (Fig. 2B).

Experiments in which a fixed number of DU-145 cells were co-cultured with variable numbers of HL-60 neutrophils revealed increased uptake of vitamin C by the DU-145 cells as the number of HL-60 neutrophils present in the assay increased (Fig. 2C). There was a 13-fold increase in vitamin C uptake, from 0.4 to 5.2 nmol per million prostate cells when the number of HL-60 neutrophils in the assay was increased from 0.1 to 5 million cells, but no further increase in uptake was observed when the HL-60 neutrophils were increased up to 30 million per assay. Parallel experiments revealed a similar correlation between the number of activated HL-60 neutrophils added to the co-culture assay and the amount of superoxide anions generated (Fig. 2D). Superoxide dismutase abolished at least 75% of vitamin C uptake by the DU-145 cells co-cultured with activated HL-60 neutrophils, indicating that superoxide is central to vitamin C uptake by the bystander cells (Fig. 2E).

Moreover, cytochalasin B, but not cytochalasin E, inhibited in a dose-dependent manner the uptake of vitamin C by the DU-145 cells co-cultured with activated HL-60 neutrophils, with greater than 95% inhibition observed at 10 μM cytochalasin B (Fig. 2F). Replacing the NaCl in the medium with choline chloride failed to affect the uptake of vitamin C by the DU-145 cells (data not shown).

Both the inhibitory effect of cytochalasin B and the sodium independence of the overall uptake process are consistent with the bystander cells taking up dehydroascorbic acid through glucose transporters. It is therefore possible that the capacity of the DU-145 cells to take up dehydroascorbic acid could be subjected to regulation by the co-culture with HL-60 neutrophils or by activation with PMA. Short uptake experiments revealed that DU-145 cells cultured alone, in the absence of PMA, transported dehydroascorbic acid at a rate of 0.83 nmol/106 cells × min, rate was unchanged in cells treated with PMA and also in cells co-cultured with HL-60 cells with or without PMA (data not shown), indicating that the capacity of the DU-145 cells to transport dehydroascorbic acid was not affected by PMA or the co-culture with HL-60 neutrophils. Long uptake studies to measure accumulation of vitamin C revealed that for all conditions tested, uptake increased in a linear manner for the first 30 min of incubation, with an average uptake rate of 0.55 nmol/106 cells × min and approached saturation at about 60 min (data not shown). Thus, PMA treatment or co-culture with the HL-60 cells did not affect the accumulation of vitamin C by the DU-145 cells. We next identified the chemical form of vitamin C accumulated intracellularly by the DU-145 cells. HPLC analysis revealed the presence of only ascorbic acid accumulated intracellularly, with more than 97% of the radioactivity accumulated by the cells eluting in the position of ascorbic acid (data not shown). We conclude that human prostate tumor cells transport the dehydroascorbic acid that is generated by locally activated cells through the glucose transporters and subsequently accumulate ascorbic acid intracellularly.

We tested the general applicability of the bystander effect by
co-culturing activated HL-60 neutrophils with a number of human primary cells and cell lines. This group of cells included normal mammary epithelial cells and the breast cancer cell lines MCF-7 and MDA-468. These cells do not express functional ascorbic acid transporters and take up dehydroascorbic acid through glucose transporters (data not shown). When incubated in the presence of activated HL-60 neutrophils, the breast cells showed increased uptake of vitamin C at 60 min, they contained an amount of vitamin C that was 6- to 20-fold higher than that accumulated by PMA-treated cells incubated alone (Fig. 3A). Moreover, cytochalasin B, but not cytochalasin E, completely blocked the uptake of vitamin C by the co-cultured bystander cells, with greater than 90% inhibition observed at 10 μM cytochalasin B (Fig. 3A and data not shown).

The bystander effect was also observed when PMA-activated fresh human neutrophils were co-cultured with the human endothelial-like cell line ECV304, the human melanoma cell line GMEL, and human umbilical vein endothelial cells (HUVEC), co-cultured with (+) or without (−) normal human neutrophils. Uptake of 50 μM ascorbic acid was tested in the absence (−) or in the presence (+) of 0.5 μM PMA, and with (+) or without (−) 20 μM cytochalasin B. Cells cultured alone in medium containing ascorbic acid without PMA were used as controls. Uptake was measured at 60 min.

B. vitamin C uptake by the endothelial cell line ECV-304, the melanoma cell line GMEL and human umbilical vein endothelial cells (HUVEC), co-cultured with (+) or without (−) normal human neutrophils. The uptake of 50 μM ascorbic acid was tested in the absence (−) or in the presence (+) of 0.5 μM PMA, and with (+) or without (−) 20 μM cytochalasin B. Cells cultured alone in medium containing ascorbic acid without PMA were used as controls. Uptake was measured at 60 min.

C. effect of glucose on vitamin C uptake by DU-145 cells co-cultured with HL-60 cells. The incubation medium contained 50 μM ascorbic acid, 0.5 μM PMA and the indicated concentrations of glucose. Uptake was measured at 60 min. D, effect of glucose on superoxide production in co-cultures of DU-145 cells and HL-60 cells. PMA was used at 0.5 μM, and superoxide production was determined at 60 min. Data are the mean ± standard deviation of one experiment performed in quadruplicate. Abbreviations: PMN, normal human neutrophils; HMEC, normal human breast epithelial cells; CytB, cytochalasin B.

Because the bystander effects involves the transport of locally generated dehydroascorbic acid through facilitative glucose transporters, we tested whether glucose, at concentrations within the expected ranges found in vivo, could block the bystander effect by directly competing for the glucose transporters. These experiments revealed that 15 mM glucose decreased by less than 40% the uptake of dehydroascorbic acid by the bystander cells (Fig. 3C), indicating that the bystander effect occurs efficiently in the presence of physiological concentrations of glucose. This less-than-expected effect of glucose on vitamin C uptake can be explained by the observation that glucose increases the production of superoxide anions by the activated cells in a dose-dependent manner, with a maximal activation of near 100% at 15 mM glucose (Fig. 3D).

**DISCUSSION**

The capacity of human neutrophils and mononuclear phagocytes to generate oxidants has been widely documented (19, 30), and these cells accumulate increased amounts of ascorbic acid when incubated under conditions that lead to the activation of the oxidative burst (15, 20–22). The data have been previously analyzed in the context of the recycling of vitamin C by activated cells, and based on the evidence that the glucose transporters are highly efficient transporters of dehydroascorbic acid, it has been suggested that the increased uptake probably represents the transport of locally generated dehydroascorbic acid (15, 22). The detailed analysis presented here is consistent with this interpretation and furthermore, we argue for a more general role of the oxidative burst in modulating vitamin C availability. Our central proposal is that the dehydroascorbic acid generated by activated cells producing superoxide can be transported by other cells present in the immedi-
Vitamin C Recycling

...ate area. We tested this hypothesis using an experimental system consisting of activated cells (HL-60 neutrophils and normal human neutrophils) co-cultured with adherent cells (bystander cells). The co-culture experiments confirmed that activated cells are able to provide vitamin C, as dehydroascorbic acid, to adjacent bystander cells by oxidizing ascorbic acid to dehydroascorbic acid, which is then taken up by the adjacent cells and immediately reduced back to ascorbic acid intracellularly.

The triggering of the oxidative burst has been classically linked to the physiological mechanism of host defense that has as its central purpose the elimination of microorganisms by strong oxidants generated by cells of the host defense system (30). Our present findings indicate that the oxidative burst leads not only to increased accumulation of vitamin C by the activated cells but also by neighboring cells via a novel bystander effect. Because of the ubiquitous expression of glucose transporters, all cells have potentially the capacity to acquire vitamin C through the bystander mechanism described here. Thus, our present findings add an additional level of complexity to previous interpretations by revealing a certain balance between oxidative and anti-oxidative actions in inflammatory reactions whereby extracellular oxidation itself causes increased intracellular concentrations of ascorbic acid. The importance of this concept lies in the fact that oxidants produced by activated phagocytes to kill microorganisms also damage adjacent tissues and cells. Similarly, oxidant damage to tissues resulting from autoimmune reactions is due in part to host defense cell activation. On the other hand, the antioxidative properties of vitamin C make it likely that an increased intracellular content of vitamin C in the adjacent bystander cells enhances their antioxidant defense mechanisms. Thus, the bystander effect links antioxidant cellular defense with extracellular oxidation.

Our data directly address the apparent paradox that although all human cells have the capacity to transport oxidized vitamin C, dehydroascorbic acid represents a very small fraction of total ascorbate in vivo (2, 31). Moreover, in vitro transport studies with isolated cells have revealed the existence of sodium-ascorbic acid co-transporters in a number of cells of animal origin (4, 6, 18, 27, 32–34). Two isoforms with the expected properties for sodium-ascorbic acid co-transporters were recently cloned from rat and human RNA, and Northern and in situ hybridization analysis revealed the presence of the mRNA in small intestine, liver, kidney, adrenal glands, brain, retina, and other tissues (35–37). Using in vitro transport assays under strictly controlled conditions to avoid the uncontrolled oxidation of ascorbic acid, we have shown absence of functional ascorbic acid transporters in a number of cells of human origin (10–12, 15). We have recently extended this analysis to normal and neoplastic cells and cells lines derived from human prostate and breast (this study) and found that they transport dehydroascorbic acid efficiently through facilitative glucose transporters. Furthermore, our studies in vivo suggest that oxidized vitamin C may cross the blood-brain barrier (16). Why, then, if all human cells express dehydroascorbic acid transporters, is there no detectable dehydroascorbic acid in blood? We reason that this may be related to the chemical properties of the different forms of vitamin C. Although ascorbic acid appears to be stable in vivo, in solution dehydroascorbic acid undergoes hydrolysis with an estimated half-life of less than 1 min (10, 38–41). The hydrolysis is an irreversible process, which if it were to occur in vivo, would imply a vast consumption of vitamin C. This is an issue of physiological significance because humans are unable to synthesize vitamin C and must obtain the vitamin from external sources in the diet. The absence of vitamin C in the diet leads to the development of scurvy with its associated problems and ultimately death. Although there has been some controversy regarding the definition of the optimal daily requirements of vitamin C in humans in exact physiological terms (42, 43), it is clear that small daily amounts of vitamin C in the diet are sufficient for the maintenance of a “normal” human physiology. It is therefore evident that the salvage of vitamin C in vivo through continuous recycling could be central to the maintenance of low daily requirements of the vitamin. This is an issue that has been addressed in the case of the recycling of vitamin C by human erythrocytes and its effect on the antioxidant reserve of whole blood (44, 45).

Our present data extend these previous observations by indicating that the dehydroascorbic acid generated locally by oxidation of ascorbic acid can be immediately transported intracellularly and reduced back to ascorbic acid by neighboring cells. Moreover, our in vitro data indicate that the bystander effect is operative in the presence of physiological concentrations of glucose, and we have preliminary evidence indicating that the bystander effect may be functional in vivo (46). Thus, the bystander effect may provide cells with an efficient system for the recycling and salvage of vitamin C.

Although the study presented here refers to human cells, its conclusions are generally applicable to cells that express glucose transporters and are involved in oxidative events of the kind described here. A mechanism for the acquisition of vitamin C based on the local oxidation of ascorbic acid is not necessarily restricted to cells of the host defense system. Although the NADPH oxidase of phagocytes has been the most thoroughly studied, there is evidence indicating that non-phagocytic human cells express a membrane oxidase with the capacity to generate superoxide. Endothelial cells, smooth muscle cells, retinal epithelial cells, and fibroblasts generate low levels of superoxide, and the activity of the oxidases expressed in these cells is regulated in a cell-dependent manner by growth factors and other cell-specific stimuli (47–49). It has been proposed that the generation of superoxide by non-phagocytic cells may be central to the delivery of signals important for cell function. Based on the results presented here, we propose that the NADPH oxidase present in non-phagocyte cells may play a role in regulating not only their own cellular uptake of vitamin C, but may also potentially modulate the uptake of vitamin C in other cells.

Our data may provide useful insights into the underlying role of vitamin C deficiency in human disease. Patients with insulin-dependent and non-insulin-dependent diabetes mellitus show an impairment of endothelium-dependent vasodilation that can be restored with vitamin C administration (50), and it has been proposed that hyperglycemia may contribute to impaired vascular function through production of superoxide anions (51). Moreover, diabetes and inflammatory disease are often accompanied by a decrease in plasma and intracellular ascorbate levels and by alterations in plasma ascorbate-dehydroascorbate ratios (52). In this context, our data are compatible with the concept that hyperglycemia may lead to decreased intracellular regeneration of ascorbic acid because of irreversible loss of extracellular dehydroascorbic acid.

In conclusion, we propose a model for vitamin C recycling that resolves the apparent contradiction that although dehydroascorbic acid is only a minor fraction of the total content of ascorbate in vivo, all human cells express dehydroascorbic acid transporters. In this model, superoxide generated by activated cells undergoing the oxidative burst oxidize extracellular ascor-
bic acid to dehydroascorbic acid which is then transported through glucose transporters by the activated cells themselves as well as other cells present in the immediate area. The transported dehydroascorbic acid is immediately reduced back to ascorbic acid, which accumulates at high concentrations intracellularly. This recycling model may work as an efficient system for the salvage of vitamin C by avoiding the irreversible hydrolysis of the dehydroascorbic acid produced in inflammatory reactions. Moreover, it provides cells with increased intracellular concentrations of ascorbic acid with its associated antioxidant properties, therefore linking normal oxidative events and protecting cells from oxidative stress. This recycling model may work as an efficient transport mechanism for the salvage of vitamin C by avoiding the irreversible hydrolysis of the dehydroascorbic acid produced in inflammatory reactions. Moreover, it provides cells with increased intracellular concentrations of ascorbic acid with its associated antioxidant properties, therefore linking normal oxidative events and protecting cells from oxidative stress.
Recycling of Vitamin C by a Bystander Effect
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