Induction of Cystine Transport Activity in Human Fibroblasts by Oxygen*  

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The transport activity for cystine in cultured human fibroblasts decreased after incubation of the cells under a low oxygen concentration. After the incubation for 48 h under 3% oxygen, the V_{max} of the transport was decreased to less than one-third of that of the control cells, with little change in K_{m}. The similar transport activity was observed in the cells cultured under 3% oxygen for 10-40 days with several times of passages. When these low oxygen-cultured cells were incubated under room air, the activity was enhanced with a lag of about 4 h and was almost completely restored within 24 h. This restoration required protein synthesis. The cystine transport activity increased by 50% after exposure of the cells to hyperoxia (40% oxygen). From these results it is concluded that the transport activity for cystine is induced by oxygen. In contrast, little change in the transport activities for alanine and leucine occurred in the cells exposed to the corresponding hypoxia or hyperoxia. Since the cystine transported into the cells is reduced to cysteine and the cysteine readily exits to the culture medium where it autoxidizes to cystine, a cystine-cysteine cycle across the plasma membrane has been postulated. Since the autoxidation of cysteine in the culture medium was markedly slowed down under the low oxygen concentration, the change in the cystine transport activity in response to the oxygen concentration was regarded as pertinent. Induction of the cystine transport activity may constitute a protective mechanism against the oxidative stress, to which the culture cells are exposed, by providing the cells with cysteine which is mainly incorporated into glutathione.  

The transport of amino acids is a cell membrane function sensitive to environmental changes. We have described in cultured human fibroblasts a Na"+-independent, anionic amino acid transport system highly specific for cystine and glutamate (1). This system, designated as System x_c (2), is an exchange agency and cystine is transported in an anionic form in exchange for glutamate (3). Since the intracellular pool of cystine is negligibly small, whereas that of glutamate is usually very large, the physiologic flows via this system are the entry of cystine and the exit of glutamate. Cystine, once it enters the cell, is rapidly reduced to cysteine. The transport of cysteine across the plasma membrane is mediated by neutral amino acid transport systems, primarily by System ASC in human fibroblasts (4). System ASC and other amino acid transport systems of mammalian cells generally mediate both the inward and outward transport (5). As a consequence of this bidirectional function of the transport systems, cysteine constantly exits from the cell because it easily autoxidizes to cystine in the extracellular fluid. Thus we can see a cycle consisting of the entry of cysteine, the intracellular reduction of cystine to cysteine, the exit of cysteine, and the extracellular oxidation of cysteine to cystine. This cycle is driven not only by the cellular activities for the transport and metabolism of cystine and cysteine, but also by the redox state in the extracellular milieu. In this paper evidence showing the regulation of the cystine transport activity by oxygen will be presented.  

EXPERIMENTAL PROCEDURES  
Materials—L-[3,3',3H]Cystine and L-[2,3,3H]alanine were obtained from Amersham International Corp. L-[2,3,4,5,5H]-Leucine was from ICN Corp. Glucose oxidase was from Wako Chemical Co., Japan.  
Cell Culture—The cells used were human diploid fibroblasts derived from fetal lung (strain IMR-90). They were grown in Eagle's basal medium supplemented with 10% fetal bovine serum. Routine subcultivations were carried out every 3-4 days with a split ratio of 1:2. For the experiments, cells plated at 1 x 10^6 in 35-mm diameter dish or at 2.5 x 10^6/60-mm diameter dish were incubated for 2 days (unless otherwise stated) before the experiment. For the hypoxic or hyperoxic culture, cells in the culture vessels were placed in the humidified airtight incubation chamber (capacity = 16 liters), and were gasped with either 3 or 40% oxygen mixture with 5% CO_2 and balance nitrogen. The chamber was maintained in an incubator at 37 °C and were regassed every 24 h. The oxygen concentration in the chamber was checked with an oxygen electrode. Some later experiments were carried out using an O_2-CO_2 incubator (Hirasawa CPO2-171, Japan).  
Uptake of Amino Acid—Uptake of cystine, alanine, and leucine was measured as described previously (1). Cells in a 35-mm diameter dish were rinsed three times in warm phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 0.01% CaCl_2, 0.01% MgCl_2·6H_2O, 0.1% glucose, and 10 mM phosphate, pH 7.4). They were then incubated in 0.5 ml of the warmed uptake medium for a specified time period at 37 °C. The uptake medium contained the H-labeled amino acid (0.05 mM and 1 μCi/0.5 ml) in the same phosphate-buffered saline used to rinse the cells. The incubation was terminated by rapidly rinsing the dish three times in 1.5 ml of ice-cold phosphate-buffered saline not containing CaCl_2, MgCl_2, and glucose. The radioactivity taken up by the cells was determined as described. The rates of uptake were determined under conditions approaching initial rates, i.e. by taking the value for the 2-min uptake of cystine and for the 0.5-min uptake of alanine or leucine.  
Determination of Sulphydryl Concentration in the Medium and GSH Content in the Cell—Sulphydryl concentration in the culture medium was measured with 5,5'-dithiobis-(2-nitrobenzoic acid) as described previously (6). GSH in the cells was measured enzymatically by the method of Tietze (7) as described previously (8).  

RESULTS  
Effect of Changing Ambient Oxygen Concentration during Culture on the Cellular Uptake of Cystine—Table I shows the
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Cells were cultured as indicated and the uptake of 0.05 mM L-[3H]cystine was measured for 2 min. The data are the means ± S.D. of six determinations.

| Rate of uptake of cystine (nmol/min/mg protein) | Preculture | Culture condition for the experiment |
|-----------------------------------------------|------------|-------------------------------------|
| 0.99 ± 0.22                                   | Under room air | Under room air for 2 days |
| 0.57 ± 0.10                                   | Under room air for 2 days | Under 3% oxygen for 1 day |
| 0.28 ± 0.05                                   | Under 3% oxygen for 8 days with 2 passages | Under 3% oxygen for 2 days |
| 0.23 ± 0.04                                   | Under 3% oxygen for 37 days with 9 passages | Under 3% oxygen for 2 days |
| 0.21 ± 0.04                                   | Under 3% oxygen for 37 days with 9 passages | Under 3% oxygen for 2 days |

rate of the uptake of cystine by the cells cultured under low oxygen concentration for various time periods. When the cells were cultured under 3% oxygen for 2 days, the uptake of cystine was depressed and the rate of the uptake fell down to nearly one-fourth of that in the cells cultured under room air. In the cells cultured for a long term under 3% oxygen, further decrease in the rate of the uptake was very small. These long term cultured cells were examined for the restoration of the uptake of cystine (Fig. 1). The cells were plated in the dish and incubated for 2 days under 3% oxygen. They were then exposed to room air and after various time periods, the rate of the uptake of cystine was measured. The uptake began to be enhanced at 6 h after exposure and was almost completely restored within 24 h to the level of the control cells cultured under room air. This restoration was completely blocked if cycloheximide, an inhibitor of the protein synthesis, was present.

Kinetic analysis of the cystine uptake was carried out to see whether the decrease in the rate of uptake was due to a decrease in $V_{max}$ and/or due to changes in $K_m$. Rate of the cystine uptake at various concentrations were measured and the double-reciprocal plots are shown in Fig. 2. The plots were linear at the cystine concentrations up to 0.5 mM, which was close to the solubility limit of cystine. From Fig. 2, the $V_{max}$ values of 2.5 and 0.77 nmol of cystine/min/mg of protein were obtained for cells cultured under room air and those cultured for 2 days under 3% oxygen, respectively. In contrast, the $K_m$ values for cystine were nearly equal (approximately 0.08 mM) in both cells.

The above results suggest that the carrier protein for the cystine uptake turns over with half-life of a day or so and that its synthesis is regulated in some way by ambient oxygen, although the possibility that oxygen could be inactivating a pathway for carrier degradation is not excluded. It should be noted that no significant difference in cell growth was observed between cultures under room air and those under 3% oxygen in our experimental system. This similar observation was reported years ago showing that at relatively higher inoculum sizes no difference in growth rate of human fibroblasts was found between cultures gassed with atmospheric oxygen and those gassed with 1% oxygen (9).

The uptake of other amino acids, alanine and leucine, was examined in the cells cultured for 2 days under low (3%), normal (20%), or high (40%) oxygen concentration, and the results, together with those for cystine, are summarized in Table II. It seems clear that the changing ambient oxygen concentration had little influence on the uptake of either cystine uptake turns over with half-life of a day or so and that its synthesis is regulated in some way by ambient oxygen, although the possibility that oxygen could be inactivating a pathway for carrier degradation is not excluded. It should be noted that no significant difference in cell growth was observed between cultures under room air and those under 3% oxygen in our experimental system. This similar observation was reported years ago showing that at relatively higher inoculum sizes no difference in growth rate of human fibroblasts was found between cultures gassed with atmospheric oxygen and those gassed with 1% oxygen (9).

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Changes in the Rates of Uptake of Alanine, Leucine, and Cystine in the Cells Cultured under Hypoxia or Hyperoxia

Routinely cultured cells were plated in the dishes and cultured for 2 days under the oxygen concentration indicated. The uptake of 0.05 mM L-[3H]alanine and L-[3H]leucine was measured for 0.5 min, and the uptake of 0.05 mM L-[3H]cystine was measured for 2 min. The data are the means ± S.D. of six determinations.

| Oxygen concentration in culture (%) | Rate of uptake of alanine (nmol/min/mg protein) | Rate of uptake of leucine (nmol/min/mg protein) | Rate of uptake of cystine (nmol/min/mg protein) |
|---|---|---|---|
| 3 | 7.40 ± 0.14 | 9.24 ± 0.70 | 1.45 ± 0.30 |
| 20 | 7.79 ± 0.21 | 9.08 ± 0.25 | 0.99 ± 0.18 |
| 40 | 6.05 ± 0.48 | 9.06 ± 0.59 | 0.25 ± 0.03 |

FIG. 3. Generation of sulfhydryl groups in the culture medium under room air. O, the cells previously cultured under 3% oxygen for several passages were plated in the dishes and cultured for 2 days under 3% oxygen. Then, the medium was changed to the fresh one and the dishes were transferred to the incubator under room air oxygen. At the time indicated, the sulfhydryl concentration in the medium was measured. * Control cells cultured under room air were treated similarly as described above, but they were always cultured under room air. The data are means ± S.D. of four determinations.

alanyl or leucine, although the uptake of alanine was slightly depressed in the cells cultured under hypoxia. The rate of the uptake of cystine in the cells cultured under 40% oxygen was increased by 50% in comparison with that in the cells under room air.

Generation of Sulfhydryl Groups in the Culture Medium—The sulfhydryl content of a stock culture medium is usually very low because sulfhydryl groups of cysteine readily autoxidize and those of serum proteins are masked by mixed disulfide formation with cysteine present in the medium. Previously we have shown that sulfhydryl groups were generated in the culture medium when such cells as human fibroblasts were cultured (6). This has been interpreted as the result that the cells took up cysteine, reduced it intracellularly, and released a reduced product, cysteine, into the medium. We have shown in the present experiment that sulfhydryl groups are generated in the culture medium by the cells. The cells previously cultured under room air generated much more sulfhydryls than the cells previously cultured under hypoxia. Most probably the difference in the capacity of the cells for generating sulfhydryl groups is ascribed to the different capacity for taking up cysteine. The autoxidation of cysteine in the medium under room air or 3% oxygen. No cells were included in the medium in this experiment. The initial rate of the decrease of sulfhydryl groups under 3% oxygen was about one-half of that under room air. Thus, when the cells were cultured under hypoxia, both the generation and the extinction of sulfhydryls seemed to be slowed down.

We measured sulfhydryl concentrations in the medium, in which the cells had been cultured for 2 days under room air or 3% oxygen. The values obtained were 30.3 ± 4.3 mM for the culture medium under room air and 25.4 ± 1.6 mM for the one under 3% oxygen. The difference is very small and apparently the sulfhydryl concentration in the culture medium is kept constant by the cells despite changing the oxygen concentration. We have also measured cellular GSH levels in the same experiments. The GSH content of the cells cultured under

Fig. 4. Autoxidation of the sulfhydryl groups of cysteine in the culture medium. O, cysteine was added (final concentration 100 μM) to the medium previously equilibrated with 3% oxygen in culture dish without cells. Then, the medium was incubated at 37 °C under 3% oxygen and at the time indicated, the concentration of sulfhydryl groups in the medium was measured. * Control experiment incubated under room air. The data are means ± S.D. of four determinations.

Fig. 5. Effect of oxygen concentration on the induction of the cystine transport activity by various stimuli. Hatched bars, the cells previously cultured under 3% oxygen for several passages were plated in dishes and cultured for 2 days under 3% oxygen (control). For the last 24 h in the culture, the cells were exposed to the cystine-free medium (-Cystine) and/or 0.1 mM diethyl maleate (+DEM); for the last 42 h in the culture the cells were exposed to 2.5 millimolar/mg glucose oxidase (+Glucose oxidase). They were then assayed for the uptake of L-[3H]cysteine (0.05 mM). Open bars, the cells routinely cultured under room air were used and treated similarly as described above, but they were always cultured under room air. The data are means ± S.D. of four determinations.
**Induction of Cystine Transport Activity by Oxygen and Other Stimuli**—The activity for the cystine uptake or cystine transport in human fibroblasts has been known to be inducible by starvation in cystine or by electrophilic agents (10). The uptake of cystine is enhanced when the cells are cultured in cystine-free medium, or when the cells are cultured in the presence of an electrophilic agent such as diethyl maleate. Because in the present study it has been shown that oxygen is also the inducing agent, we have investigated a possible relation between oxygen and other stimuli. The results are summarized in Fig. 5. When the cells were cultured in cystine-free medium, the cystine uptake was potently enhanced but the effect of oxygen as the inducing agent for the cystine uptake was almost extinguished. In contrast, diethyl maleate enhanced the cystine uptake leaving the effect of oxygen unchanged. When the two effects overlapped, i.e. when the cells were incubated in cystine-free medium containing diethyl maleate, the cystine uptake of these cells was much more enhanced and the effect of oxygen disappeared. The results suggest that, in the induction of the cystine uptake, the effects of oxygen and the starvation for cystine are interrelated but the effect of diethyl maleate is practically independent. Because the starvation for cystine causes a serious depletion of the intracellular glutathione (8), it is highly likely that the cells are exposed to severe oxidative stress even though the ambient oxygen concentration is lowered. We have measured also the oxygen effect in the presence of glucose oxidase, which reacts with glucose in the medium and produces hydrogen peroxide. Under room air the uptake of cystine was enhanced slightly by glucose oxidase, whereas under 3% oxygen the uptake was enhanced strongly by glucose oxidase. Thus the apparent effect of oxygen became less marked if glucose oxidase was present.

**Discussion**

Maintenance of normal cell function in the presence of oxygen depends on effective protecting mechanisms (11). Oxygen-derived free radicals are continuously produced in the cells since the fixation of one electron onto the oxygen molecule during cellular metabolism produces superoxide anion followed by the formation of hydrogen peroxide, hydroxyl and peroxyl radicals. These activated oxygen species are very unstable and reactive. They attack lipids leading to the dysfunction of membrane, proteins and nucleic acids leading to the structural and functional disturbance, and finally to cell death. For the protection against these harmful molecules, cells are provided with several defense systems. Among them, GSH and its related enzymes are of particular importance (12). Glutathione peroxidase reduces organic and hydrogen peroxides into the corresponding alcohols using GSH as substrate. GSH is synthesized in two steps catalyzed by γ-glutamylcysteine synthetase and GSH synthetase, respectively. Although mammalian cells are provided with these enzymes to synthesize their own GSH and the GSH functions in a various manner in the cell, its degradation is believed to occur outside the cell (13). GSH or GSSG is transported out and degraded in such an organ as kidney, where the degradation enzymes are localized. Thus GSH are constantly lost from the cell and the availability of the precursor amino acids, especially cysteine, is an important factor in the regulation of cellular GSH level.

The feature of cysteine distinctive from other amino acids is that the sulfhydryl form cysteine is predominant inside the cell, whereas the disulfide form cystine is predominant outside the cell. Cultured human fibroblasts absolutely require cystine in the medium for maintaining the GSH level and for the survival (8). In these cells cystine is transported into the cells almost exclusively through the cystine-glutamate exchange system, or System x meticulate cycle is illustrated in Fig. 6. Since the oxidation of cysteine occurs constantly in extracellular fluid, cells have to drive this cycle continuously to maintain the intracellular level of cysteine, which is the rate-limiting precursor for GSH synthesis. Thus, although the cycle is apparently futile, its role is unequivocal. The present study has shown that the activity of the cystine transport or System x meticulate cycle is inducible by oxygen. Since the formation of cystine in the cycle is accelerated by the increase in oxygen concentration, the induction of the cystine transport activity by oxygen is fully significant. The direct driving force of this cycle is a steep concentration gradient of glutamate (14). The major source of the intracellular glutamate is glutamine, present in the culture medium abundantly.

The mechanism by which oxygen induces the activity is not known at present. However, it is highly likely that activated oxygen molecules are involved because hydrogen peroxide is the effective inducer (Fig. 5). Diethyl maleate is a potent inducer of the System x meticulate cycle is inducible by oxygen. Since the formation of cystine in the cycle is accelerated by the increase in oxygen concentration, the induction of the cystine transport activity by oxygen is fully significant. The direct driving force of this cycle is a steep concentration gradient of glutamate (14). The major source of the intracellular glutamate is glutamine, present in the culture medium abundantly.

**System x meticulate** is almost ubiquitous in cultured cell lines (15). However, it was absent in isolated hepatocytes (16), isolated kidney cells (17), and peritoneal macrophages (18), although it emerged soon after they were cultured under routine culture conditions (in room air). Probably cells in culture are exposed to the oxidative stress more severely than those in vivo. The activity of System x meticulate found in primary cultured cells may result from the induction by the oxidative stress. Hepatocytes...
can synthesize cysteine from methionine sulfur, and all mammalian cells so far studied have neutral amino acid transport systems and can take up cysteine via these systems (19). However, cystine is not a neutral (dipolar) amino acid and obviously it has to be utilized because it is continuously formed by the autoxidation of cysteine. At present we do not know how widespread System $x^{-}$ is in vivo and to clarify this remains to be challenged. Recently the activity similar to that of System $x^{-}$ has been found in the brain synaptosomal fraction (20, 21). Presumably cystine formed in the extracellular fluid of brain hardly goes back to blood due to the blood-brain barrier (22) and may be utilized by the brain cell through this system.

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