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LIPOFUSCIN ACCUMULATION IN CULTURED NON-DIVIDING CELLS AS A FUNCTION OF TIME AND OXYGEN TENSION

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Abstract

Cultivated human glial cells, kept in a state of density-dependent inhibition of growth, accumulate age-pigment (lipofucsin) within their lysosomal vacuomes with the same characteristics as the corresponding pigment observed *in vivo*. The rate of formation and accumulation of lipofuscin is greatly accelerated under the conditions of routine cell cultivation in comparison to the *in vivo* event. Lipofuscin is generally considered to be composed of polymerized products of lipid peroxidation and thus it would be reasonable to suggest that factors which influence lipid peroxidation would also alter the rate of lipofuscin formation. Human glial cells were grown in the presence of various oxygen concentrations in the gas-phase (5%, 10%, 20%, 40%). This was found to modulate (accelerate or decrease) the rate of lipofuscin formation. The present study thus provides: (1) important supportive evidence for the lipid peroxidation origin of lipofuscin, (2) a useful model system for studying the effect of lipofuscin accumulation on lysosomal function and cell growth kinetics, (3) evidence that our standard culture conditions are far from ideal since oxygen concentration may drastically alter rates of lipofuscin formation and accumulation. Cell culture technique, as we know it today, may benefit from more closely controlled oxygen tensions, i.e., by reducing oxygen to levels that more closely approximate conditions *in vivo*.

KEY WORDS: Cultured cells (human), glial cells, pigments, lipofuscin, fluorescence measurements, free radicals, lipid peroxidation, oxygen.

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Introduction

Lipofuscin is a brownish pigment with yellow-green autofluorescence which consists of cross-linked polymers of polyunsaturated fatty acids and protein residues which accumulate within the lysosomal vacuome (total volume of secondary lysosomes) of post-mitotic cells during their life-time *in vivo* (Armstrong 1985). It is considered to be the end-product of a free radical induced cross-linking of proteins with peroxidized lipids (Tappel 1975) including those present in the membranes of organelles sequestered within the lysosomes during autophagocytosis (Collins et al. 1980).

Accumulation of lipofuscin normally occurs quite slowly, as a function of age, but at quite different rates in various species, and even within different post-mitotic cells (e.g., nerve cells) of the same species. The deposition of these lipid peroxidation products may be accelerated greatly by nutritional or vitamin deficiencies, cytotoxic agents or in certain pathological conditions such as neuronal lipofuscinosis (Zeman and Siakotos 1973).

It has been reported that lipofuscin granules, similar to those found in post-mitotic cells *in vivo*, accumulate in routinely cultivated diploid cells, especially during density-dependent inhibition of growth (Brunk and Collins 1981). The material thus formed within the cultivated cells has the same biophysical characteristics, localization and electron microscopical appearance as the lipofuscin found *in vivo* (Collins and Brunk 1976).

Growth inhibition of human glial cells *in vitro* (weeks) results in a rapid accumulation of lipofuscin pigment (Brunk and Collins 1981), an accumulation which *in vivo* would normally occur during a life (years), which suggests that the *in vitro* culture conditions, as we know them today, are far from ideal.

Oxygen is an important prerequisite to the survival of most cells, but it is also recognized as potentially toxic to all forms of life. The role that oxygen plays in regulating normal

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cellular metabolism is not well understood, but its toxicity at concentrations significantly greater than the ambient fifth of an atmosphere has been clearly documented (Fridovich 1975; Jones 1985). In the current view, some biological reduction of oxygen occurs through a pathway (Forman and Boveris 1982) which gives rise to reactive oxygen metabolites; the superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). Subsequently hydroxyl radicals ($OH^{\cdot}$) may be formed through metal catalysis if $O_2^{\cdot-}$ and H_2O_2 are not efficiently scavenged. It has been shown that these oxygen metabolites are all reactive species and could not be endured, were it not for the elaborate defences that cells have necessarily evolved. They combine special enzymatic scavenging systems such as superoxide dismutase, catalase, and glutathione peroxidase with low molecular weight antioxidants such as vitamin E and glutathione (Halliwell 1981). These defences are of varied nature, sufficient to handle ordinary needs, but may easily become overwhelmed when the delicate oxidative balance of the cellular milieu is altered.

It is thus conceivable that variations in oxygen tension may influence the rate of formation and scavenging of reactive oxygen species, which may in turn alter the rate of lipid peroxidation, and subsequently the formation of lipofuscin. We have thus studied the role of various oxygen tensions in the gas phase of the cell culture system on the formation rate of lipofuscin in individual cultivated human glial cells, following formation of stable, density-dependent growth-inhibited cell monolayers.

Materials and Methods

Cell lines and culture conditions

The experiments were performed on a line of U-787 CG diploid, normal human glial cells cultivated *in vitro*, derived and maintained in culture as described earlier (Pontén et al. 1969). The cells were pooled and plated in the 23rd passage at a concentration of 6000 cells/cm^2 in 75-ml Nuclon^R plastic bottles, nourished with Eagle's minimal essential medium (EMEM) supplemented with 10% new born calf serum and antibiotics, and grown in a humidified environment of 20% O_2: 75% N_2: 5% CO_2 at 37°C. The medium was changed twice weekly until the cells reached maximal confluency and ensuing density-dependent inhibition of growth (aproximately 21 days).

Some of the above density-dependent growth-inhibited cultures were then placed in air-tight chambers (Flow Laboratories, Svenska AB, Solna, Sweden) which permitted the strict control of oxygen tension using various gas mixtures (AGA Specialgas, Lidingö, Sweden) and were then grown under the following gaseous environmental conditions: (a) 5% O_2: 90% N_2: 5% CO_2; (b) 10% O_2: 85% N_2: 5% CO_2; (c) 40% O_2: 55% N_2: 5% CO_2. Control cells were grown in ordinary cell culture chambers using air (20% O_2: 75% N_2) and 5% CO_2. In the following text these gas mixtures will be referred to in terms of their O_2 content. The EMEM (as above) was changed twice weekly. For each medium change the EMEM was first gassed with the appropriate O_2 mixture and the newly changed cultures were placed in the incubators which were first gassed and then sealed. Cell cultures were prepared for analysis at 3, 6, 9, and 12 weeks following initiation of growth under the different oxygen concentrations, as described below.

Preparation for microscopical analysis

Cell cultures were exposed to 0.25% collagenase (Worthington, type I) for 2h at 37°C and then trypsinized for subcultivation as described earlier (Collins and Thaw 1983). They were seeded (4000 cells/cm^2) into tissue culture chambers (Lab-Tek Products, USA), re-incubated in EMEM for settling during an additional 6h, rinsed, and then fixed overnight in 4% formaldehyde in 0.15 M sodium cacodylate HCl buffer (pH 7.4). Following removal of the chambers, the slides were rapidly rinsed in distilled water, air dried and mounted under coverslips using Entellan^R (E. Merck).

Measurement of autofluorescence

The cells were individually localized in the phase contrast mode of a Leitz MPV-II microscope fluorometer. Care was taken to include only those cells that appeared well outstretched and isolated. The autofluorescence of the individual cells was then measured (minus background fluorescence) using an HBO 100 high-pressure Hg lamp, a 3 mm BG12 excitation filter and a K530 barrier filter. A uranyl standard was used for calibration of the microscope. Data values were stored, collated and sorted using an ABC 800 (Luxor AB, Motala, Sweden) computer which was integrated with the MPV-II microscope. The computer system, comprising both hardware and a comprehensive special applications software package, has been described earlier (Rundquist 1981).

Results

Controls (20% O_2)

The distribution of autofluorescence values at different time periods is shown in Fig 1. Autofluorescence per cell increased by 420% from an initial median value of 106 arbitrary units (a.u.) to over 550 a.u. after three months.

Influence of 5%, 10%, and 40% O_2 tension

The medians of autofluorescence per cell (a.u.) at 3, 6, and 12 weeks are shown in Fig 1. The median autofluorescence per cell was consistently lower than controls in cell cultures exposed to either 5% or 10% O_2, in proportion to the decrease in O_2 tension. Those cultures grown in an environment of 40% O_2 showed a large increase in autofluorescence per cell as compared to the control cell population after all time periods.

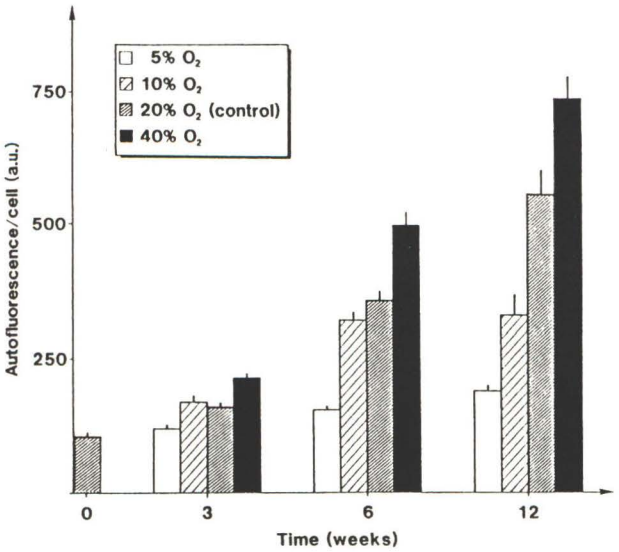

Fig 1: Time (weeks). Ordinate: a. u.(arbitrary units) indicating median autofluorescence / cell for cells grown under the indicated conditions and studied at different times following density dependent inhibition of growth. Bars indicate standard error of the mean. n = 200 cells in each group.

Discussion

The relationship between lipofuscin-induced autofluorescence and the quantity of lipofuscin has received much attention (Dowson and Harris 1981), and has recently been supported by correlative analysis using both electron-microscopical (stereological) and fluorescence (autofluorescence) techniques to measure lipid peroxidation products (lipofuscin) in individual cultivated human glial cells (Collins and Thaw 1983).

Methodological problems concerning the techniques used in this study exist and it is possible that some of the procedures may have resulted in an overall underestimation of the lipofuscin content of some cells, since the percentage of not fully spread cells appears to increase in relation to lipofuscin content (i.e., quenching may occur in cells which have not fully spread). Subjectively, there were no apparent differences observed in the settling of cells exposed to the various treatments.

That lipofuscin may be formed within cells as a result of autoxidation of polyunsaturated lipid and proteins, with subsequent cross-linking and polymerization, has been suggested on the basis of *in vitro* experiments (Dillard and Tappel 1971). Such oxidations involve a series of self-propagating reactions giving rise to highly toxic intermediates, some of which are free radicals.

It has been suggested that the rate of formation of peroxidation products (lipofuscin) is a reflection of the total peroxidative processes occurring within the cytoplasm and the lysosomal vacuome. It may thus be reasonable to suggest that factors influencing redox processes within the cell will also influence the formation rate of lipofuscin. Pro-oxidants such as high oxygen tension accelerate the rate of lipofuscin formation, while reduced oxygen tension retard lipofuscin formation rate.

An objective comparison of the lipofuscin accumulation velocity between the *in vivo* (years) and *in vitro* (weeks) situations suggest that standard culture conditions are far from ideal. The growth of cell cultures at various oxygen tensions demonstrates that the formation of lipid peroxidation products (lipofuscin) can be slowed down or speeded up by altering the exposure of cells to oxygen. It has previously been demonstrated that low oxygen tension enhances the period of replicative growth for normal cells in culture (Packer and Fuehr 1977) and it is thus conceivable that reducing oxygen in the gas phase during routine cell culture might be beneficial and may more closely approximate physiological conditions for cell growth.

The different treatments of our cell cultures, designed to vary the levels of lipid peroxidation, have resulted in markedly varied rates of lipofuscin accumulation. This supports the notion that lipofuscin is derived from cellular lipid peroxidation products. A comparison of cellular oxygen (Siesjö 1978) tensions of normal brain tissue (3-4% O_2) with the oxygen concentration used in the gas phase of today's routine culture systems (20% O_2) gives rise to some interesting questions about our culture conditions and the relevance of some conclusions drawn from some studies of cultured cells.

The effect of lipofuscin on cell function remains unclear (Brizee and Ordy 1981), although some investigations point to its rather marginal importance in the hampered cellular function seen with age *in vivo*. It may, however, influence the function of the lysosomal vacuome to some degree and also reflect the level of lipid peroxidation and the state of its controlling or scavenging mechanisms within the cell. It would thus be of great interest to investigate how altered lipid peroxidation levels affect heterophagocytosis, autophagocytosis and cell culture growth kinetics (i.e., phase III phenomenon).

Acknowledgements

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Discussion with Reviewers

B.A. Afzelius: In view of your findings, do you think the low lipofuscin accumulation at low oxygen partial pressure may be a partial explanation to the fact that some of the most long living tribes known live in the Andes and in the Himalayas?
Authors: This is a fascinating idea to which I think there is no certain answer. It would certainly be of great interest to study lipofuscin accumulation in neurons from people of old age living in these areas.

B. Forslind: In the introduction you say that the material formed within the cultivated cells has the same biophysical characteristics etc as lipofuscin found in vivo. What characteristics are you referring to?
Authors: Autofluorescent property and electron density as seen in the transmission electron microscope.

B. Forslind: Your results on the effect of the oxygen tension at cell culture is most interesting and obviously they have impact on any kind of aerobic cell culture. Have you any suggestion of how one should optimize the oxygen tension for a specific tissue? How do you assess the in vivo oxygen of a particular tissue?
Authors: We have no experience on other cells than human glial cells and we have not measured in vivo oxygen tensions.

G.M. Roomans: Were any statistical tests done on the data to determine the degree of significance of the oxygen effect?
Authors: Statistical tests according to Neushal-Wallis and Mann-Whitney were carried out and showed the differences to be significant, as is clearly evident from Fig 1.