Purines induce lipofuscin formation in a colon carcinoma cell line

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Lipofuscin was produced when HT29, a colon carcinoma cell line, was cultured in millimolar concentrations of xanthine and guanine but not in the presence of other bases. Using a simple assay developed to quantify the fluorescent pigment, it was found that maximum levels of lipofuscin were developed in 3 days. Methylxanthines that are not substrates of xanthine dehydrogenase, such as caffeine and theophylline, did not induce

formation of lipofuscin. Xanthine-induced lipofuscin formation could be inhibited by oxypurinol, indicating that the pigment may be formed by free radicals generated by xanthine dehydrogenase. It is suggested that the lipofuscin seen in pseudomelanosis coli may result from the accumulation of purines in the colon.

INTRODUCTION

Lipofuscin accumulates slowly in man over long periods of time, its increased incidence in older individuals giving rise to the term ageing pigment. It is thought to be a heterogenous complex formed between proteins and peroxidized lipids (Tsuchida et al., 1987; Harman, 1989) and, as such, lipofuscin is considered to be a marker of oxidative stress (Sohal and Brunk, 1989). It has been suggested that the reactive oxygen species involved in its production may leak from mitochondria (Brunk et al., 1992) or be generated by macrophages (Hendriks and Eestermans, 1986; Walker et al., 1988; Maeba et al., 1990). Particularly large accumulations of the pigment may occur in lipofuscinosis coli, where it is mostly observed in macrophages, although it probably originates in epithelial cells of the mucosa that are subsequently removed by phagocytosis (Ghadially and Parry, 1966; Walker et al., 1988).

Lipofuscinosis coli, previously called pseudomelanosis coli, is a benign pathological condition characterized by varying degrees of large-bowel pigmentation, that has been recognized for a long time (Stewart and Hickman, 1931). The chemical nature and origins of the pigment found in the colon and rectum of such patients has been controversial. Although theories have included melanin produced from aromatic amino acid degradation products and complexes between anthraquinones and tissue components, histochemical and electron microscopic analysis have led to the pigment being recognized now as a lipofuscin (Pearse, 1985; Walker et al., 1988). Lipofuscinosis coli is usually associated with abuse of anthraquinone-containing laxatives (Speare, 1951; Ghadially and Parry, 1966; Badiali et al., 1985). In a recent study, however, 60% of the large bowels investigated microscopically at autopsy were found to contain pigment granules (Koskela et al., 1989). Such a high frequency of lipofuscinosis coli is inconsistent with the incidence of laxative abuse and suggests a different aetiology. The origin of the oxidative damage generating lipofuscin in the colon, however, has not been identified. Results shown here, obtained after chance observations with a colon carcinoma cell line, suggest that lipofuscin may be formed by xanthine oxidase activity generated by exposure to high concentrations of purines.

MATERIALS AND METHODS

Chemicals

Bases and oxypurinol (obtained from Sigma Chemical Company, Poole, Dorset, U.K.) were prepared as 40 mM stock solutions, titrated with 1 M NaOH to dissolve, and stored frozen at –20 °C. Allopurinol (Zyloric, Wellcome Foundation, U.K.) was dissolved in distilled water according to the manufacturer's instructions. *N*-Acetylcysteine, glutathione, 2,6-dichlorophenol-indophenol (DCPIP), superoxide dismutase and catalase were obtained from Sigma and dissolved in PBS (Oxoid, Unipath Ltd., U.K.). All solutions were filtered through 0.2 µm pore size filters (Ministart NML, Sartorius GmbH, Germany). Superoxide dismutase and catalase were dissolved immediately before use.

Cell culture

The human colon carcinoma cell line HT29 (Fogh and Trempe, 1975) was routinely cultured in αMEM (ICN Flow, High Wycombe, Bucks., U.K.) with a final glucose concentration of 1 g/l and with 10% (v/v) added heat-inactivated newborn bovine serum, without antibiotics, in 90-mm-diam. tissue culture dishes (Falcon 3003, Becton Dickinson). In some experiments, toxic effects of chemicals on cell growth were measured by a dye-binding assay (Winterbourne, 1986).

Lipofuscin quantification

HT29 cells were seeded at 1×10^5 cells per well in 24-well cluster plates and grown under the required conditions. When present, cells containing lipofuscin were seen irregularly distributed throughout the culture, with a significant number of cells floating in the medium. To quantify the lipofuscin, a reliable sampling method was developed. The cells were harvested by direct sonication in the medium within the well (15–20 s at a power setting of 40 at 25 W, Bioblock Scientific, Vibra Cell 72434, model ASI probe). Lipofuscin-containing granules were collected by centrifugation in microcentrifuge tubes (5 min, 11600 *g*), the well was washed with 1 ml of PBS, and the wash was added to the pellet, which was resuspended by vortexing and recentrifuged. The pellet was resuspended in 200 µl of PBS by sonication as

Abbreviation used: DCPIP, 2,6-dichlorophenolindophenol.

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Figure 1 Pigment in HT29 cells grown in medium containing xanthine

Phase contrast (a) and fluorescence (b) microscopy of cells remaining attached after 5 days in 2 mM xanthine. The cell sheet was washed three times in PBS, to remove floating aggregates of lipofuscin that made photography difficult, and fixed for 10 min in 3.4% formaldehyde in PBS before microscopy (bar = 200 μ m).

above, for 3–5 s. For each sample a 25 μ l aliquot was deposited as a 1-mm-diam. spot by filtration through a Whatman GF/D glass-fibre filter supported on absorbent paper, using a pipette tip with a 1-mm-wide orifice. The filter was baked dry at 80 °C and assembled between two microscope slides. Fluorescent light output was measured with a Nikon UFX II in a Nikon Optiphot microscope, using 450–490 nm excitation with a 510 nm dichroic mirror and a 520 nm barrier filter. An arbitrary measure of light output was calculated as 100/(exposure time in s).

RESULTS

Growth of HT29 cells for several days in high concentrations of xanthine resulted in the appearance of dark granular material. The pigment was acid fast in the Ziehl–Neelsen stain and gave the characteristic yellow-green fluorescence of lipofuscin when excited with blue light (Pearse, 1985) (Figure 1). No fluorescent pigment was detected in cells not exposed to xanthine (results not shown). The extent to which lipofuscin granules were formed was variable between cells in a single dish and many aggregates of lipofuscin, presumably from dead cells, were seen floating in the medium. These factors made it difficult to quantify the response.

Attempts to measure the fluorescent product in a fluorimeter were prevented by light scatter. The pigmented granules were not dissolved by detergents or by digestion with proteinase K. In agreement with other studies of lipofuscin, only small amounts of material, which had a blue fluorescence, could be extracted with chloroform and methanol (Shimasaki et al., 1977); the majority of the green fluorescent material remained in the pellet.

Figure 2 Fluorescence quantification of lipofuscin

A sample of lipofuscin-containing granules was serially diluted in PBS. Aliquots were spotted on glass-fibre filters and assayed for lipofuscin as described in the Materials and methods section. The means of triplicates are shown and the results are representative of six separate experiments.

Figure 3 Effect of xanthine concentration on lipofuscin formation

HT29 cells were grown for 5 days in various concentrations of xanthine. Each concentration was tested in triplicate wells of the 24-well culture dish. An aliquot from each well was assayed for lipofuscin as described in the Materials and methods section. The results shown are the means of triplicate wells and are representative of six separate experiments.

It was possible, however, to get a linear response with the fluorescent microscope by measuring the light output from granules collected on filters (Figure 2).

Very little lipofuscin was produced at concentrations of xanthine up to 1 mM, but the levels increased dramatically at concentrations greater than 1.5 mM (Figure 3). In the presence of 2 mM xanthine, the fluorescent pigment appeared rapidly, being detectable within 1 day and reaching a peak by about 3 days (Figure 4). In addition to causing pigmentation, xanthine was highly toxic to the cells over a similar concentration range, as shown by the effects on the colony forming efficiency of HT29 cells plated at low density (Figure 5).

When cells were cultured in other bases at 2 mM concentra-

Figure 4 Time course of lipofuscin formation

HT29 cells were seeded into the 24 wells of a cluster plate. At appropriate intervals, xanthine was added to a final concentration of 2 mM in groups of four wells. All wells were harvested at the same time, and analysed and plotted as described in Figure 3. The results correspond with visual observations in all other experiments.

Figure 5 Toxicity of xanthine to HT29 cells

HT29 cells were seeded at 100 cells per 35-mm-diam. dish in the presence of various concentrations of xanthine. After growth for 11 days, the colonies were fixed, stained with 1% Crystal Violet and counted. The means of triplicate dishes at each concentration are shown. The results are representative of two separate experiments.

tions, only guanine produced a significant amount of lipofuscin, although at a substantially lower level than cells cultured in xanthine. Other purines and pyrimidines did not induce lipofuscin in HT29 cells (Table 1). Similarly, the two methylxanthines, caffeine and theophylline, failed to generate any lipofuscin (Table 2). Oxypurinol, the specific xanthine oxidase inhibitor, reduced the amount of lipofuscin produced in the presence of 2 mM xanthine (Figure 6). Allopurinol also inhibited lipofuscin production, but was slightly less effective than oxypurinol (results not shown). Taken together, these results suggest that the lipofuscin was produced by the activity of xanthine oxidase.

Lipofuscin formation induced by 2 mM xanthine was not

Table 1 Lipofuscin production by HT29 cells grown in medium supplemented with various bases

Each base was added to cultures of HT29 in triplicate at 2 mM. After 5 days, the tests were assayed for lipofuscin fluorescence as described in the Materials and methods section and the results expressed as means of triplicates.

Base	Fluorescence (arbitrary units)
None	0.95
Adenine	1.56
Cytosine	1.06
Hypoxanthine	1.03
Guanine	8.69
Uracil	1.03
Xanthine	93.8

Table 2 Lipofuscin production by HT29 cells grown in medium supplemented with xanthine and methylxanthines

Each xanthine was added to cultures of HT29 in triplicate at 2 mM. The tests were assayed after 5 days for lipofuscin fluorescence as described in the Materials and methods section and the results expressed as means of triplicates.

Base	Fluorescence (arbitrary units)
Xanthine	96.53
Caffeine	2.48
Theophylline	2.27

Figure 6 Inhibition of xanthine-induced lipofuscin formation by oxypurinol

HT29 cells were grown in medium containing 2 mM xanthine and various concentrations of oxypurinol, in triplicate. After 3 days, the cells were harvested and assayed as described in Figure 3. The results are representative of two separate experiments.

inhibited by superoxide dismutase (up to 500 units/ml) or catalase (up to 1000 units/ml), either alone or in combination. Similarly, *N*-acetylcysteine and glutathione (up to 1 mM) were unable to block formation of the pigment. The alternative electron acceptors, DCPIP and Methylene Blue, were investigated for their ability to inhibit lipofuscin formation. Although DCPIP suppressed fluorescence at concentrations above 20 μ M (Figure 7a), this was mostly due to its toxicity (Figure 7b). Methylene

Figure 7 Effects of DCPIP on growth and lipofuscin production

HT29 cells were grown in medium containing 2 mM xanthine and various concentrations of DCPIP in triplicate. After 3 days, the cells were harvested and assayed as described in Figure 3. An additional set of triplicate wells was used to assay cell growth by Coomassie Blue staining, measured at 620 nm (Winterbourne, 1986). (a) Lipofuscin production. (b) Cell growth. The results are representative of two separate experiments.

Blue was even more toxic and the intense blue staining of the cells prevented lipofuscin quantification.

In a limited survey of five cell lines, one other, GER [a human pancreatic cell line (Grant et al., 1979)], also produced large amounts of lipofuscin when grown in medium containing xanthine. By contrast, the hamster fibroblastic cell line CHO K1, PMC42 [a human breast cancer cell line (Whitehead et al., 1983)] and secondary cultures of cells isolated from human peritoneum, showed little evidence of lipofuscin production (results not shown).

DISCUSSION

Our results show that colonic epithelial cells, after short-term exposure to millimolar concentrations of the purines xanthine or guanine, form large amounts of lipofuscin. Xanthine (which is also formed from guanine, by deamination) is converted into uric acid by the enzyme xanthine dehydrogenase. By contrast the methylxanthines, caffeine and theophylline, are not effective substrates for this enzyme (Krenitsky et al., 1972), and did not induce lipofuscin. The failure of adenine and hypoxanthine to

generate any significant quantity of lipofuscin may reflect the ready incorporation of these purines into adenine nucleotide pools under aerobic conditions (Harkness, 1988). Together with the inhibition by oxypurinol, the results suggest that the lipofuscin was formed by reactive oxygen species generated by xanthine dehydrogenase.

Under physiological conditions, the enzyme oxidizing xanthine is predominantly a dehydrogenase that uses NAD^+ as electron acceptor. However, the dehydrogenase may be converted into an oxidase (McKelvey et al., 1988; Nordback and Cameron, 1993) and even highly purified dehydrogenase exhibits a limited activity with oxygen as electron acceptor (Nishino and Tamura, 1991). Proteolytic cleavage irreversibly converts the enzyme into an oxidase, but conversion may also occur reversibly, by oxidation of sulphhydryl groups (Nishino and Tamura, 1991). It has been proposed that such conversion may be responsible for the damage that occurs on reperfusion of ischaemic tissue (McCord, 1985; Granger, 1988). According to this theory, anoxia results both in the conversion of xanthine dehydrogenase into the oxidase by a calcium-dependent protease, and in accumulation of the substrate, hypoxanthine. Subsequent reoxygenation of the tissue results in the formation of superoxide and tissue damage. In the experiments reported here, reactive oxygen species are produced intracellularly by exposure to high concentrations of purines alone, under conditions of ambient oxygen tension. Previous investigators using cultured cells to study reactive oxygen-mediated damage have added exogenous xanthine oxidase to millimolar concentrations of hypoxanthine or xanthine in the culture medium (Iwata et al., 1984; Hall et al., 1988; Paul et al., 1989; Chiricolo et al., 1991).

Although cells contain a variety of free-radical-scavenging systems, they appear to have been unable to protect against the formation of lipofuscin in these experiments. The inability to prevent lipofuscin formation by addition of exogenous antioxidants and free-radical-scavenging enzymes, may be due to the intracellular site of formation and action of the highly reactive oxidants producing the lipofuscin. By contrast with the antioxidants, low concentrations of oxypurinol, a specific inhibitor of xanthine dehydrogenase, was able to inhibit lipofuscin production. In agreement with other reports (Matsuki et al., 1990), allopurinol was found to be less effective, possibly due to the formation of some free radicals as it is oxidized by the enzyme to oxypurinol, as the final inhibitor (Spector, 1977). It has been proposed that Methylene Blue may be able to protect tissues from free radical damage by acting as an alternative electron acceptor for xanthine oxidase (Salaris et al., 1991); however, this dye was found to be extremely toxic to the cells used here. DCPIP may also accept electrons from the molybdenum in xanthine dehydrogenase (Spector, 1977) and is a competitive inhibitor in the fluorimetric assay of the enzyme (Mest et al., 1992). Although there was a suggestion of inhibition by DCPIP, particularly when surviving cells were examined microscopically, this electron acceptor was also very toxic.

These results suggest a novel hypothesis for the origins of lipofuscinosis coli: that excessive accumulation of purines in the colon results in free radicals being produced by xanthine dehydrogenase, with the subsequent formation of lipofuscin. The purines may be derived both from the diet and from endogenous sources, such as cells sloughed off from the lumen of the gastrointestinal tract. The concentration of purines in the lumen of the colon is not known, but plasma concentrations of purines, mostly obtained from fasting individuals, are in the range 0.2–5 μM , although substantial increases may occur in some conditions (Harkness, 1988). Dietary purines are generally believed to be absorbed and catabolized to uric acid in the small

intestine, and excreted in the urine (Savaiano et al., 1980). The absorption depends on the type and form of the dietary purine (Brule et al., 1988), however, and in some cases substantial amounts of the ingested purines are unaccounted for and are believed to remain in the lumen of the gut (Giesecke et al., 1982). The normal American diet results in a daily intake of about 600–1000 mg of purines (Krause and Mahan, 1984). A variety of factors, including method of cooking and extent of mastication, will determine the digestibility and absorption of the dietary purine intake. Taking account of the volume of chyle entering the colon (about 500–1000 ml), the absorption of water (resulting in a 5-fold increase in concentration during the passage through the colon), and the time of contact with a potentially non-homogeneous mixture (typically 1–3 days), it is plausible that the epithelium may be exposed to sufficiently high purine concentrations in some individuals to cause the formation of lipofuscin.

The high incidence of pigment in large bowels investigated microscopically at autopsy (Koskela et al., 1989), may reflect the purine-rich diet common to the western world. If the explanation proposed here is correct, the customary diagnosis of laxative abuse in all patients with lipofuscinosis coli may have to be re-examined. In the cases of established laxative abuse, the increased shedding of cells caused by laxatives may generate lipofuscin by the mechanism suggested above. The transient wave of apoptosis noted after anthraquinone administration (Walker et al., 1988), by increasing the release of purines in the colon, may be consistent with this proposal. Additionally, anthraquinones may have a direct effect on the conversion of xanthine dehydrogenase into the oxidase form of the enzyme.

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