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Killing of Arterial Smooth Muscle Cells by Chylomicron Remnants

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Chylomicron remnants were found to be cytotoxic to cultured arterial smooth muscle cells. Cell death was estimated by two methods: colourimetric assay using a tetrazolium salt and trypan blue staining. Both methods showed considerable cell death. Twenty-five micrograms of cholesterol/ml of chylomicron remnants appeared not to injure smooth muscle cells; however, greater concentrations (= and >50 μg cholesterol/ml) caused considerable damage. Our observations may offer a possible cause of cell death in atherosclerotic plaques.

Cell death is a feature of advanced atherosclerotic plaques. The dead cells in plaques primarily originate from smooth muscle cells and macrophages (1), however, the cause of death is unknown. Chung and coworkers found that remnants of triglyceride-rich lipoproteins from human hypertriglyceridemic serum were toxic towards macrophages (2) and endothelial cells (3) in vitro. However, they did not identify the type of remnants responsible for causing cell death. Remnants of triglyceride-rich lipoproteins are either of intestinal or hepatic origin. Circulating remnants in animals fed an atherogenic diet were suspected to cause atherosclerosis, suggesting involvement of postprandial lipoproteins, that is chylomicrons (4). In circulation, nascent chylomicrons are rapidly converted to their remnant form following hydrolysis by endothelial lipases. Mamo and Wheeler (5) showed that CR penetrated the arterial wall and were selectively retained compared to several other lipoproteins. The plasma concentration of chylomicron remnants (CR) is elevated for several hours after eating (3), therefore focal arterial accumulation of CR following a meal may exacerbate their putative cytotoxicity.

The purpose of this study was to determine whether CR are cytotoxic towards vascular cells. Here we report that cultured arterial smooth muscle cells (ASMC) exposed to CR at physiological concentrations suffered considerable damage. It is possible that CR-induced death of ASMC may explain some of the features of the pathogenesis of atherosclerosis.

MATERIALS AND METHODS

Chemicals. DMEM and FBS were purchased from Gibco BRL (Grand Island, NY, USA). BSA, Trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma (St. Louis, MO, USA). Culture flasks and plates were from Falcon (Becton Dickinson, New Jersey, USA). Cholesterol was determined colourimetrically (Trace Scientific, catalog number: TR13015, Australia). Triglycerides were determined following corrections for free glycerol (Wako, catalog numbers: 430-11291, 432-11491, 436-11391, 438-11591, Japan). Filters (0.2 μm) were from Gelman Sciences (Ann Arbor, MI, USA). Other reagents and chemicals were from Ajax (NSW, Australia).

ASMC culture. ASMC were cultured from rabbit aortas by the explant method as described previously (6). Cells cultured for 4 weeks were harvested and then subcultured in 48 well culture plates (5000 cells/well) where they were incubated with CR.

CR. Lymph chylomicrons were obtained as described previously (7). CR were prepared by injecting the chylomicrons (0.3 g of triglyceride/kg of body weight) into the blood circulation of functionally hepatectomized rabbits (4–5 kg). The chylomicrons were left to circulate for 2.5 to 3 h. CR (density < 1.006 g/ml) were isolated from rabbit plasma by

Abbreviations used: ASMC; arterial smooth muscle cells; BSA, bovine serum albumin; CR, chylomicron remnants; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid disodium; FBS, fetal bovine serum; LDL, low-density lipoproteins; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM, standard error of mean.

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ultracentrifugation, dialyzed overnight in PBS/0.05% (w/v) EDTA, and sterilized by passing through a 0.2 μm filters. CR were kept at room temperature under nitrogen and used within 2 days. Cholesterol mass was determined by a colourimetric assay. Cholesterol oxidation products (oxysterols) and free fatty acids (unesterified fatty acids) in CR were analyzed by mass spectrometry (8) and gas chromatography (9) respectively.

MTT (tetrazolium) assay for cell viability. Subcultured cells were maintained in DMEM plus 20% FBS for 6 days and a further 3 days in DMEM without FBS. The cells were incubated with CR in DMEM. At the indicated time, the cells were washed once with PBS (pH 7.4) plus 1 mg/ml BSA followed by two washes of only PBS (pH 7.4). Cell viability was determined by the MTT assay modified from Mosmann (10). DMEM containing MTT (0.5 mg/ml) was added to each well of cells and incubated for 1.5 hours at 37°C in the humidified atmosphere of 95% air/5% CO_2. An equal volume of 0.04 N HCl-isopropanol was added to each well and incubated for a further 30 min at room temperature. Absorbance of the coloured solution was read at a test wavelength of 570 nm against a reference wavelength of 630 nm.

Trypan blue staining. After incubation with chylomicron remnants, cells were detached in 0.05% trypsin/0.53 mM EDTA, washed twice in PBS (pH 7.4) at 1200 rpm for 5 min and resuspended in 0.05% trypan blue in PBS (pH 7.4). After 3 min, the number of stained cells were counted on a hemocytometer. A minimum of 200 cells were counted.

RESULTS

Figure 1 shows the effect of CR on cell viability at 20 h as determined by the MTT assay. There was no cell loss in the absence of CR or at a CR cholesterol concentration of 25 μg cholesterol/ml. However, at higher concentrations viability seemed to progressively decrease. At 50 μg/ml there was a 20% loss of cells, whilst at 100 and 150 μg cholesterol/ml, cell viability was 46.59 ± 5.45% and 38.92 ± 4.87% respectively.

The effect of incubation time on ASMC viability is illustrated in Fig. 2. CR at a fixed concentration of 150 μg cholesterol/ml exhibited time-dependent cytotoxicity with only 30.42 ± 3.43% of cells surviving at 24 h.

The trypan blue exclusion test was also used to determine cell death. Fig. 3 shows trypan blue staining of ASMC after incubation with increasing concentrations of CR at 20 h. At lower concentrations of 25 and 50 μg cholesterol/ml, the number of cells stained was less than 11%, however, at higher concentrations of 100 and 150 μg cholesterol/ml, it was more than 40%. Staining of cells exposed to CR at 150 μg cholesterol/ml as a function of time is shown in Fig. 4. At the end of the 24 h incubation, there was approximately 83.33 ± 2.6% of 'dead' cells.

Mass spectrometry with a sensitivity of 1 ng was used to detect CR cholesterol oxidation products (oxysterols). No cholesterol oxidation products were found from three separate preparations.

DISCUSSION

We used the MTT assay and trypan blue exclusion test to assess ASMC viability in the presence of postprandial remnant lipoproteins. Both methods showed consistent results indicating consid-

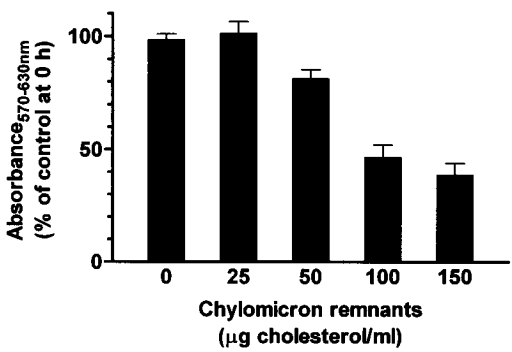

FIG. 1. Effect of increasing CR concentration on the survival of ASMC at 20 h as determined by the MTT assay. Cells were incubated in the absence or presence of CR (μg cholesterol/ml), 37°C, 20 h. Cell viability was determined by the MTT assay as described under Materials and Methods. Controls were cells with no CR at 0 h. Data are presented as mean ± SEM of three separate experiments.

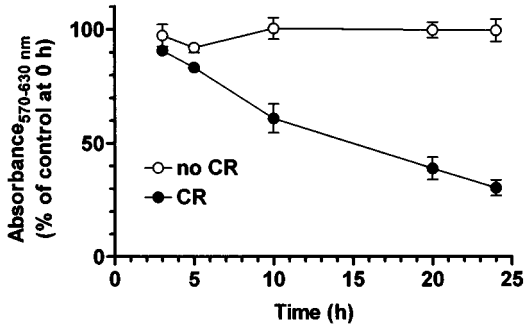

FIG. 2. Time course of ASMC viability incubated with CR determined by the MTT assay. Cells were incubated with 150 μg cholesterol/ml of CR, and at the indicated time, cell viability was determined by the MTT assay as described in the legend to Fig. 1. Data are presented as the mean ± SEM of three separate experiments.

erable death of ASMC exposed to CR. The highest CR concentration used (150 μg cholesterol/ml) was within a physiological plasma chylomicron remnant cholesterol concentration (11), however, it does not make allowances for the potential focal accumulation of CR in atherosclerotic lesions. Clearly, CR cytotoxicity was dose and time dependent, therefore in vivo, arterial cytotoxicity would presumably depend on arterial influx and efflux of remnants. The delivery of CR to arterial beds (via transcytosis) primarily reflects their concentration in plasma (5, 12). Increased remnant concentration following a meal would lead to a sharp rise in arterial delivery, possibly inducing an acute cytotoxic situation following localized accumulation. CR do not efflux from arterial tissue efficiently (5) and furthermore are selectively retained within sites of lesion formation (12), consistent with their putative atherogenicity.

Remnant-induced cytotoxicity was hypothesized by Chung and coworkers to be due to free fatty acid accumulation following hydrolysis by lipases, within the lipoprotein complexes (2, 3, 13). However, we found that the free fatty acid concentration of pre-lipolyzed nascent chylomicrons (8.55 μM/g cholesterol) was in fact higher than for CR (2.66 μM/g cholesterol). In contrast to this study, Chung and co-workers generated remnants of triglyceride-rich lipoproteins in vitro. In Chung's study, hydrolysis of hypertriglyceridemic serum in the absence of sufficient free fatty acid acceptors may have given rise to remnants artificially enriched in free fatty acids.

Low-density-lipoproteins (LDL) containing oxysterols are cytotoxic to ASMC and macrophages (14, 15). Oxidatively modified LDL might induce cell death either directly, or following stimulation of superoxide radical production (16). Alternatively Mitchinson and his colleagues found that

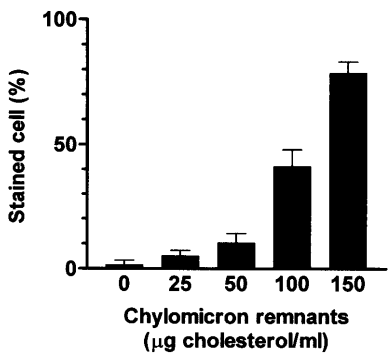

FIG. 3. Trypan blue staining of ASMC after exposure to CR at 20 h. After incubation with CR, cells were stained with 0.05% trypan blue in PBS (pH 7.4) as described under Materials and Methods. Data are presented as the mean ± SEM of three separate experiments.

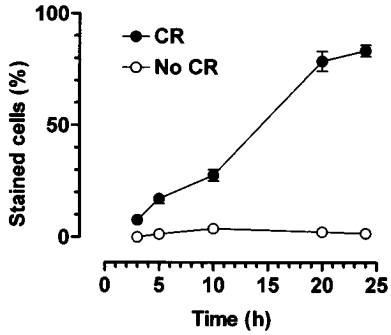

FIG. 4. Trypan blue staining of ASMC exposed to CR (150 μg cholesterol/ml) with time. At the indicated time points, after incubation with CR, cell viability was determined as described in the legend to Fig. 1. Data are presented as mean ± SEM of three separate experiments.

macrophages exposed to oxidized LDL underwent apoptosis (17). We found no detectable oxysterols in our CR preparations suggesting that cytotoxicity was because of another CR component or following activation of a metabolic pathway.

In conclusion, we have identified that physiological concentrations of CR induce cellular death of ASMC in vitro. Our observations suggest that cell death in atherosclerotic lesions might in part be due to accumulation of CR. Presently, the mechanism of CR-induced cytotoxicity of ASMC remains unresolved.

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