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Effects of apoE genotype on macrophage inflammation and heme oxygenase-1 expression

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Abstract

In order to gain a more comprehensive understanding of the aetiology of apolipoprotein E4 genotype-cardiovascular disease (CVD) associations, the impact of the apoE genotype on the macrophage inflammatory response was examined. The murine monocyte–macrophage cell line (RAW 264.7) stably transfected to produce equal amounts of human apoE3 or apoE4 was used. Following LPS stimulation, apoE4-macrophages showed higher and lower concentrations of tumour necrosis factor alpha (pro-inflammatory) and interleukin 10 (anti-inflammatory), respectively, both at mRNA and protein levels. In addition, increased expression of heme oxygenase-1 (a stress-induced anti-inflammatory protein) was observed in the apoE4-cells. Furthermore, in apoE4-macrophages, an enhanced transactivation of the key redox sensitive transcription factor NF-κB was shown. Current data indicate that apoE4 macrophages have an altered inflammatory response, which may contribute to the higher CVD risk observed in apoE4 carriers.

Keywords: apoE genotype; Macrophage; Cytokines; Nuclear factor κB; Heme oxygenase-1; Tumour necrosis factor α; Inflammation; Oxidative stress; Redox signalling

Apolipoprotein E (apoE) is a polymorphic multifunctional protein with three common isoforms in humans (E2, E3, and E4). ApoE3 is the wild-type and most common isoform, while apoE4 carriers account for about 25% of the Caucasian population [1]. Presence of the apoE4 allele is associated with a 40–50% higher risk of cardiovascular disease (CVD) [2] and apoE4 is the major known genetic risk factor for maturity-onset Alzheimer's disease (AD) [3].

Although apoE4 is strongly linked to both diseases, the molecular basis of these associations remains uncertain. Traditionally, the differential risk has been attributed to the increased low density lipoprotein cholesterol (~8%) observed in apoE4 carriers, but it is becoming increasingly evident that this alone cannot explain the disease differential [4].

ApoE is not only synthesised by the liver, but also in the brain and by resident macrophages [5] in the atherosclerotic wall, where it exerts atheroprotective actions, independent of its role in lipid metabolism [6]. These localised functions include regulation of smooth muscle cell migration and proliferation [7], inhibition of adhesion molecule expression in endothelial cells [8] and inhibition of platelet aggregation [9]. In addition to its paracrine effect on surrounding cells, apoE has also been shown to impact on macrophage function by promoting cholesterol efflux [10] and modulating NO production [11]. Inflammation and oxidative stress are key features of the pathology

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of atherosclerosis and AD. A limited number of studies, which have largely focussed on brain biology and neurodegeneration [12,13] have reported on the immuno-modulatory properties of apoE and its impact on inflammatory mediators [14]. However, little is known about the possible role of apoE genotype as a mediator of the macrophage innate immune and inflammatory responses in relation to CVD.

Using a murine macrophage cell line, which has been stably transfected with human apoE3 or apoE4, we have recently showed that apoE isoform affects macrophage oxidative status [15], and now we hypothesise that this may be accompanied by an altered inflammatory response. Furthermore, the impact of genotype on the activity of the transcription factor nuclear factor κB (NF-κB), which is known to play a major role in modulating the inflammatory response, will be presented.

Methods

Cell culture. RAW 264.7 murine macrophage cell lines, stably transfected with either human apoE3 or apoE4 were kindly given by Dr. B. Pitas (Gladstone Institute, UCSF, USA). Cells were genotyped for human apoE3 and apoE4 by the method of Hixson and Vernier [16] and apoE concentrations were measured in supernatants to ensure that secreted levels in 24 h were physiological and comparable among the two clones. Mean (SEM) levels of 1.38 (0.38) and 1.36 (0.38) μg/mg cell protein were secreted in 24 h by apoE3 and apoE4 cells, respectively, as has been previously reported [15]. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 100 μg/ml G-418. Macrophages were grown in a humidified incubator at 37 °C and 5% CO_2. Cells were incubated with lipopolysacharide (LPS) (*Salmonella enteriditis*, Sigma–Aldrich, Taufkirchen, Germany) for zdifferent time-periods depending on the outcome of interest.

Cytokine levels. Cells were stimulated with increasing concentrations of LPS (0–10 μg/ml) for 4 h. Supernatants were collected for analysis 20 h later. Cytokine concentrations were measured using commercial ELISA kits according to the manufacturer's instructions. Tumour necrosis factor alpha (TNFα) was determined by the Quantikine® mouse TNFα kit (R&D Systems, Wiesbaden, Germany), Interleukin (IL) 6 and 10 were measured using the Mouse Biotrak ELISA systems (Amersham Biosciences, Freiburg, Germany), and IL1β and macrophage inflammatory protein-1alpha (MIP1α) were determined with the mouse RayBio® ELISA kits from Ray Biotech (Norcross, USA). Values were normalised for total cell protein which was determined using the BCA assay (Pierce Biotechnology, Rockford, USA).

Cytokine and heme oxygenase-1 (HO-1) mRNA levels. Cells were stimulated with LPS (1 μg/ml) for 1 h to determine TNFα mRNA levels and for 6 h to determine mRNA levels for IL1β, IL6, IL10, and MIP1α. The time-points were chosen on the basis of maximum mRNA expression for each cytokine in a time-course experiment (data not shown). Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). One step RT-PCR was carried out using the QuantiTect®SYBR®Green RT-PCR kit (Qiagen) according to supplier instructions. For HO-1, cells were stimulated with LPS (1 μg/ml) for 24 h and RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction and reverse transcription was carried out with oligo-dT primers for 1 h at 42 °C using MMLV reverse transcriptase, according to the manufacturer's instructions (Promega, Madison, WI, USA). Real-time RT-PCR was performed with the SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). For all reactions, the Rotor Gene RG-3000 (Corbett Research) cycler was used and relative quantification of gene expression was calculated based on the $2^{-\Delta\Delta Ct}$ method (β-actin or elongation factor 2 were used as housekeeping genes). Primers and cycling conditions are shown in Table 1.

Table 1
PCR primers and conditions

	Sequence (5′–3′)	Annealing *T*
β-Actin	F: GACAGGATGCAGAAGGAGATTACT R: TGATCCACATCTGCTGGAAGGT	55
TNFα	F: CATCTTCTCAAAATTCGAGTGACAA R: TGGGAGTAGACAAGGTACAACCC	55
IL1β	F: CAACCAACAAGTGATATTCTCCATG R: GATCCACACTCTCCAGCTGCA	55
IL6	F: CTGCAAGAGACTTCCATCCAGTT R: GAAGTAGGGAAGGCCGTGG	60
MIP1-α	F: CCTCTGTCACCTGCTCAACA R: GATGAATTGGCGTGGAATCT	55
IL10	F: GGTTGCCAAGCCTTATCGGA R: ACCTGCTCCACTGCCTTGCT	60
EF2	F: GCGGTCAGCACAATGGCATA R: GACATCACCAAGGGTGTGCAG	58
HO-1	F: GTGGAGACGCTTTACGTAGTGC R: GACATCACCAAGGGTGTGCAG	58

Note. F, forward primer; R, reverse primer; IL, interleukin; TNFα, tumour necrosis factor α; MIP1α, macrophage inflammatory protein 1 α; EF2, elongation factor 2; HO-1, heme oxygenase-1.

Transcription factor activity. The NF-κB-secretory alkaline phosphatase (NF-κB-SEAP) (Clontech, BD Biosciences, Palo Alto, USA) reporter construct was used to measure the binding of transcription factors to the κ enhancer, and the activation of this pathway. Cells growing in 24 well plates were transiently transfected with 0.5 μg of the vector by SuperFect® transfection Reagent (Qiagen) according to the manufacturer's protocol. Twenty-four hours later, cells were stimulated with varying concentrations of LPS (0–1 μg/ml). At 6, 12, and 24 h, the cell culture media was removed and stored for analysis. The chemiluminescent SEAP assay (Clontech) was carried out as has been previously described [17]. Values were normalised for total cell protein determined by the BCA assay (Sigma).

Western blot analysis for heme oxygenase-1 (HO-1). Total cellular protein was isolated using ice-cold lysis buffer (1× PBS, 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM leupeptine, 10 mM aprotinin and 1% Triton X-100). Samples were centrifuged (10 min, 8000*g*, 4 °C) and clear supernatants were collected. Twenty-five micrograms of protein was loaded on 12% SDS–PAGE gel followed by transfer to nitrocellulose membrane PROTRAN (Perkin-Elmer Life Sciences, Boston, USA). Following overnight blocking (4 °C in 5% non-fat milk), membranes were probed with polyclonal antibodies against HO-1 (Stressgen Biotech, Canada) and monoclonal antibodies against α-tubulin (both diluted 1:1000 in TTBS with 3% albumin) at room temperature for 1.5 h followed by anti-rabbit HRP-linked secondary antibodies (Cell Signalling Technology, USA) (1:10,000 in TTBS with 3% albumin) for 40 min at room temperature. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) according to the manufacturer's instructions.

Statistical analysis. Statistical calculations were conducted with SPSS Version 13.0. *T*-Tests (for independent samples) were performed to compare the outcomes between apoE3 and apoE4 cells. In the absence of normal distributed data, Mann–Whitney *U*-test was used. Results are expressed as means ± SEM and significance was accepted at $P < 0.05$.

Results

Cytokine protein and mRNA levels

Stimulation of cells with increasing concentrations of LPS (0–10 μg/ml) resulted in a dose–response accumula-