Inflammation response at the transcriptional level of HepG2 cells induced by multi-walled carbon nanotubes

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Abstract. Poor information are currently available about the biological effects of multi-walled carbon nanotubes (MWCNT) on the liver. In this study, we evaluated the effects of MWCNT at the transcriptional level on the classical in vitro model of HepG2 hepatocarcinoma cells. The expression levels of 96 transcript species implicated in the inflammatory and immune responses was studied after a 24h incubation of HepG2 cells in presence of raw MWCNT dispersed in water by stirring. Among the 46 transcript species detected, only a few transcripts including mRNA coding for interleukine-7, chemokines receptor of the C-C families CCR7, as well as Endothelin-1, were statistically more abundant after treatment with MWCNT. Altogether, these data indicate that MWCNT can only induce a weak inflammatory response in HepG2 cells.

1. Introduction

Depending on the publications, parameters influencing the carbon nanotubes (CNT) toxicity include surface structure [1-3]; length [4]; agglomeration state [5,6], presence of a dispersing agent [7-10]; and contamination with metal catalyst and amorphous carbon [11]. Being single-walled or multi-walled, is also crucial to determine the biological effects of CNT. Consideration of the cell line tested also seems important when dealing with the toxic potential of CNT. We have shown previously that multi-walled carbon nanotubes (MWCNT) can be toxic and pro-inflammatory for telomerase-immortalized human keratinocytes [7,8]. However, MWCNT exerted no cytotoxicity on SV-40 immortalized sebocytes and in vitro reconstituted epidermises [7]. Moreover, MWCNT did not modify Caco-2 cells or HepG2 cells viability and did not induce release of pro-inflammatory interleukin-8 (IL-8) [12] (unpublished data). MWCNT were nevertheless able to abnormal induce production of reactive oxygen species (ROS) in HepG2 cells (unpublished data). Overproduction of ROS seems to be an important biological effect of CNT [9] even in absence of cell death [11]. Still the pro-oxidant effect has to be confirmed and deeper investigated to determine the possible mechanisms leading to ROS overproduction. Indeed, other studies showed that MWCNT did not induce oxidative stress [13] or were a remarkable radical scavengers when in contact with an external source of hydroxyl or superoxide radicals [14]. Oxidative stress can contribute to the initiation and progression of chronic...
inflammation by promoting cell proliferation, expression of adhesion molecules and matrix metalloprotenase and production of cytokines/chemokines. Oxidative signals are able to modulate the expression of a variety of cytokines and chemokines, such as Tumor Necrosis Factor-α (TNF-α), IL-1β, IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1), and adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [15]. In this study, we investigated the effect of MWCNT on the level of 96 transcript species implicated in the inflammatory and immune responses after a 24h incubation of HepG2 cells in presence of raw MWCNT dispersed in water by stirring.

2. Materials and Methods

2.1. MWCNT characterisation and dispersion
Physico-chemical characterisation of MWCNT (Nanocyl\textsuperscript{TM} NC7000 multi-walled carbon nanotubes, from Nanocyl s.a.) was described previously [7,16]. Briefly, MWCNT have an average diameter of 12 nm and variable lengths in the micron range. Their specific surface area is very high (323.7 m\textsuperscript{2}/g). They are mainly composed of carbon but presence of catalytic metal residues was detected (Al 2.1%, Fe 0.1%, Co 0.1%) [7, 16]. Raw MWCNT were dispersed into pure water at 1 mg/ml by stirring with a magnetic bar for 90 min. MWCNT suspension were then diluted at 100 µg/ml into cell culture medium before incubation of the cells for 24h. The size distribution (calculated as hydrodynamic diameters) of MWCNT suspended in culture medium was analysed by centrifugal sedimentation using a Disc Centrifuge DC24000 (CPS) at 24,000 rpm. MWCNT formed big agglomerates with an average size of 3.8 µm.

2.2. Cell culture
Human hepatocarcinoma cells (HepG2) were maintained in culture (20 passages) in 75-cm\textsuperscript{2} polystyrene flasks (Costar) with 15 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 5 ml/500 ml penicillin-streptomycin (BioWhittaker) and 10% fetal calf serum and incubated under an atmosphere of 5% CO\textsubscript{2}.

2.3. Taqman low density array
Total RNA from biological triplicate was extracted using the "TRI Reagent Soln" (Ambion). mRNA contained in 1 µg of total RNA was reverse transcribed using the "High-Capacity cDNA Reverse Transcription" kit (Applied Biosystems). Then multiplex qPCR amplifications were performed with an ABI PRISM 7000 SDS thermal cycler, using 384-well "microfluidic card" plates (Human Immune Array, Applied Biosystems) containing various primer pairs for parallel analyses of 96 transcripts on 4 samples. GAPDH mRNA was used as the reference for normalization and relative mRNA steady-state level quantification based on cycle thresholds (Ct). Melting curves were generated after amplification, and data were analyzed using the thermal cycler SDS 2.2.2 software by the $2^\Delta\Delta$C\textsubscript{t} method [17]. CTL mean values were calculated from triplicate ct values for each mRNA. Each MWCNT-treated triplicate value was then calculated by the $2^\Delta\Delta$C\textsubscript{t} method in comparison with the CTL mean value and S.D. were determined. When calculable, statistical significance was considered for P < 0.05 using Student’s t-tests.

3. Results
The possibility of a pro-inflammatory response of HepG2 cells incubated for 24h with MWCNT was investigated in triplicate by reverse transcription (RT) real time TaqMan PCR low density arrays (TLDA). These arrays allow the quantitative TaqMan PCR amplification of 96 transcripts involved in human immunity and inflammation. Among the 46 transcript species detected (listed in Table 1), only a few transcripts were statistically more abundant after treatment with MWCNT (Table 2). In this case, 3 independent biological replicates could be considered with a mean ratio calculated with standard deviations (s.d.). High variability is frequently obtained when profiling gene expression in
triplicates with arrays [18,19]. Other changes of abundance were not statistically significant when large or lacking (mean ratios calculated from only two values) s.d. were obtained despite all efforts. A third type of data are qualitative data, representing situations where a transcript is not abundant enough and below the detection threshold in the basal state, but activated upon treatment with the MWCNT [19]. In the case of LRP2 the ratio obtained from two quantitative values is indicated, the third value being qualitative. Consideration of these three types of data is equally important since they may represent a tip for further functional studies at protein level. We observed an increased abundance of transcripts of pro-inflammatory cytokines or proteins able to recruit and activate immune cells, such as interleukine-7 (IL-7), chemokines receptors of the C-C families (CCR7, CCR4), as well as colony stimulated factor-3 (CSF3) or lymphotoxin alpha (LTA). IL-8 was among all the transcript whose expression level is not modified following MWCNT exposure (data not shown), confirming previous other studies [11].

4. Discussion

Poor data are currently available on the impacts of CNT at the hepatic level. Only a few studies described the effects of MWCNT on HepG2 cells demonstrating poor or no cytotoxic effects [12].

In this study, we investigated the effects of MWCNT on the abundance of transcripts coding for proteins implicated in the inflammatory and immune responses. Several transcripts of pro-inflammatory cytokines or proteins able to recruit and activate immune cells were overexpressed after an incubation of 24h in presence of MWCNT. For example, the abundance of IL-7, chemokines CCR7 and CCR4 and CSF3 transcripts was increased after incubation with MWCNT. CCR4 and CCR7 belong to the superfamily of G-protein-coupled receptors. These chemokine receptors are membrane-bound proteins composed of 7-transmembrane domains and are coupled to G-proteins (for a review [20]). After interaction with their specific chemokine ligands, chemokine receptors trigger a release of calcium ions (Ca\(^{2+}\)) from intracellular stores leading to the activation of protein kinase C (PKC) and other calcium-sensitive protein kinases. This causes cell responses, including the onset of a process known as chemotaxis that traffic the cell to a desired location within the organism. Recently, CCR7 was shown to be involved in the survival of metastatic squamous cell carcinoma of the neck and head [21] and to promote migration and invasion of some tumours [22]. By inducing its expression, MWCNT could favor the survival of the hepatocarcinoma HepG2 cell line.

IL-7 is a cytokine important for B and T cell development [23] which is described to be produced by hepatocytes in normal adult human liver forming a suitable microenvironment for T cell differentiation [24]. This cytokine and the hepatocyte growth factor form a heterodimer that functions as a pre-pro-B cell growth-stimulating factor.

Endothelin-1 (EDN1) is a key mediator of vascular tone and renal homeostasis through antagonistic vasoactive effects. EDN1 induce vasoconstriction by binding to its receptor ET\(_A\) associated with vascular smooth muscle in the arteries walls. It has also a vasodilation property on endothelial cells by inducing the production of the potent vasodilator, nitric oxide (NO) following its binding with the ET\(_B\) receptor (for a review [25]). EDN1 plays also an important role in wound healing in a variety of parenchymal organs including liver [26].

Low density lipoprotein-related protein 2 (LRP2 or megalin) is a multiligand binding receptor found in the plasma membrane of many absorptive epithelial cells. LRP2 plays important role in the regulation of levels of diverse circulating compounds like vitamins A and D and cholesterol homeostasis by receptor-mediated endocytosis of lipoprotein particles.

LTA is a member of the tumor necrosis factor family (also called TNF-β) produced mainly by lymphocytes mediating a large variety of inflammatory, immunostimulatory, and antiviral responses. Interestingly, LTA was shown to mediate both hepatocyte and progenitor oval cell-associated liver regeneration after acute or chronic liver injuries respectively [27].
Table 1. Alphabetic list of the 46 transcripts detected in HepG2 cells by RT real time TaqMan PCR array analysis, including one transcript used as housekeeping gene for data normalisation (GAPDH).

Gene symbols are given with full gene names and general functions of the encoded proteins.

| Symbol | Name | Function |
|--------|------|----------|
| ACE    | angiotensin I converting enzyme (peptidyl-dipeptidase A) | Renin-angiotensin system regulation |
| ACTB   | actin, beta | Cell cytoskeleton and mobility |
| AGTR1  | angiotensin II receptor, type 1 | Vasoconstriction, aldosterone synthesis |
| BAX    | BCL2-associated X protein | Apoptosis |
| BCL2   | B-cell CLL/lymphoma 2 | Apoptosis |
| BCL2L1 | BCL2-like 1 | Apoptosis |
| C3     | complement component 3 | Complement system activation |
| CCL5   | chemokine (C-C motif) ligand 5 | Basophil and eosinophil activation - Chemoattraction |
| CCR4   | chemokine (C-C motif) receptor 4 | C-C chemokines receptor - Immune signal transduction |
| CCR7   | chemokine (C-C motif) receptor 7 | C-C chemokines receptor - Immune signal transduction |
| CD3E   | CD3E antigen, epsilon polypeptide (Tt3 complex) | T cell receptor - CD3 complex - Immune transduction |
| CD4    | CD4 antigen (p55) | Immune response - Antigen presentation |
| CD8A   | CD8 antigen, alpha polypeptide (p32) | Immune response - Antigen recognition |
| CD40   | CD40 antigen (TNF receptor superfamily member 5) | TNF receptor - Immune transduction |
| CD68   | CD68 antigen | Macrophage recruitment and activation |
| COL4A5 | collagen, type IV, alpha 5 | Basement membrane structure |
| CSF1   | colony stimulating factor 1 (macrophage) | Macrophage differentiation |
| CSF3   | colony stimulating factor 3 (granulocyte) | Granulocyte differentiation |
| CYP7A1 | cytochrome P450, family 7, subfamily A, polypeptide 1 | Drug metabolism and cholesterol catabolism |
| ECE1   | endothelin converting enzyme 1 | Endothelin processing |
| EDN1   | endothelin 1 | Vasooconstriction and vasodilatation |
| FAS    | Fas (TNF receptor superfamily, member 6) | Apoptosis |
| FASLG  | Fas ligand (TNF superfamily, member 6) | Apoptosis |
| FN1    | fibronectin 1 | Cell adherence - Connective tissue structure |
| GAPDH  | glyceraldehyde-3-phosphate dehydrogenase | Glycolysis |
| GUSB   | glucuronidase, beta | Glycosaminoglycan metabolism |
| HMOX1  | heme oxygenase (decycling) 1 | Heme catabolism - Anti-oxidant defenses |
| ICAM1  | intercellular adhesion molecule 1 (CD54) | Cell adherence |
| IKKB   | inhibitor of NFkB, kinase beta | NF-kappaB regulation |
| IL7    | interleukin 7 | B and T cell development |
| IL8    | interleukin 8 | Inflammation - Chemoattraction - Angiogenesis |
| IL12A  | interleukin 12A | T cell differentiation, T cell stimulating factor, anti-angiogenic activity |
| IL18   | interleukin 18 | T cell and B cell activation |
| LRP2   | low density lipoprotein-related protein 2 | Uptake of lipoproteins, sterols, vitamin-binding proteins and hormones |
| LTA    | lymphotixin alpha (TNF superfamily, member 1) | Inflammation, apoptosis, immune and antiviral responses |
| NFKB2  | nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 | Inflammation - Anti-oxidant defenses |
| NOS2A  | nitric oxide synthase 2A (inducible, hepatocytes) | Cell signal transduction |
| PGK1   | phosphoglycerate kinase 1 | Glycolysis |
| PRF1   | perforin 1 (pore forming protein) | Cell death, apoptosis, cell membrane pore-forming protein |
| RPL3L  | ribosomal protein L3-like | Ribosomal protein |
| SKI    | v-ski sarcoma viral oncogene homolog (avian) | TGF-beta signaling repression |
| SMAD3  | SMAD, mothers against DPP homolog 3 (Drosophila) | Cytokine signaling |
| SMAD7  | SMAD, mothers against DPP homolog 7 (Drosophila) | Cytokine signaling |
| STAT3  | signal transducer and activator of transcription 3 | Apoptosis - Cell cycle |
| TFRC   | transferrin receptor (p90, CD71) | Iron metabolism |
| TGFB1  | transforming growth factor, beta 1 | Cell cycle - Differentiation - Cell adherence |
| TNF    | tumor necrosis factor (TNF superfamily, member 2) | Cell cycle - Differentiation - Apoptosis |
| VEGF   | vascular endothelial growth factor | Angiogenesis - Vascular permeability |
| 18S    | 18S ribosomal RNA | Ribosomal RNA |
Table 2. Results of the transcriptomic profiling (RT real time TaqMan PCR arrays) of HepG2 cells considering 96 transcripts involved in immunity or pro-inflammatory response, after treatment of 24 h with 100 µg/ml of raw H2O-stirred MWCNT. Results were expressed as the ratios of the levels of transcripts in treated cells versus untreated cells (mean ratios ± s.d. for n = 3). Ratios between 0.7 and 1.3 were cut off. The means lacking s.d. values were obtained from 2 quantitative ratios. When a given mRNA was detected in MWCNT-treated cells and was below the detection threshold in the untreated cells, these ratios were qualified as qualitative. Grey boxes indicate statistically significant effects with P < 0.05.

| mRNA  | Mean  | S.D.  |
|-------|-------|-------|
| FASLG | 16.62 |       |
| LTA   | 15.08 |       |
| CCR4  | 4.00  |       |
| LRP2  | 3.96  |       |
| CSF3  | 3.64  |       |
| ACE   | 3.18  |       |
| CCR7  | 2.92  | 1.06  |
| EDN1  | 2.91  | 0.47  |
| IL-7  | 1.45  | 0.17  |
| CCL5  | 0.59  |       |
| CD8A  | 0.21  |       |

Currently, almost no data are available concerning the putative role of these different proteins in MWCNT-treated HepG2 cells. Moreover, these data indicate altogether that MWCNT can only induce a weak inflammatory response in HepG2 cells. Indeed, among the 46 detected mRNA, only these few transcripts were shown to be more abundant in comparison with the control cells. By comparison, water-stirred MWCNT induced overexpression of 26 transcripts coding for immune and inflammatory proteins in telomerase-immortalised human keratinocytes [8]. Furthermore, IL-8, which is among the main cytokines produced during a hepatic inflammation [28] did not show a significant increase of the abundance of their mRNA, confirming previous observation [11]. Recently, subcutaneously injected oxidated water-soluble MWCNT were shown to act as an immunostimulatory substance in the tumor-bearing mice. MWCNT induced significant activation of the complement system, promoted inflammatory cytokines production and activated macrophage cells leading to the inhibition of the tumor growth. A similar inflammatory response was not observed in our study. The origin of MWCNT (pristine MWCNT in our study and oxidized MWCNT in Meng et al. [29]) is likely to explain these differences. Oxidation is a process which modifies MWCNT surface and decreases their length [30]. These alterations can in turn change the biological effects of MWCNT. In addition, the experimental context (in vitro or in vivo) is also an important parameter to explain these differences in inflammatory responses induced by MWCNT.

In conclusion, this preliminary study described the impact of MWCNT at the transcriptional level on hepatocarcinoma HepG2 cell line showing very few effects of MWCNT on expression of mRNA coding for immune and inflammation proteins.

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