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Transforming activities of *Chlamydia pneumoniae* in human mesothelial cells

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Summary. Knowledge in viral oncology has made considerable progress in the field of cancer fight. However, the role of bacteria as mediators of oncogenesis has not yet been elucidated. As cancer still is the leading cause of death in developed countries, understanding the long-term effects of bacteria has become of great importance as a possible means of cancer prevention. This study reports that *Chlamydia pneumoniae* infection induce transformation of human mesothelial cells. Mes1 cells infected with *C. pneumoniae* at a multiplicity of infection of 4 inclusion-forming units/cell showed many intracellular inclusion bodies. After a 7-day infection an increased proliferative activity was also observed. Real-time PCR analysis revealed a strong induction of calretinin, Wilms' tumour gene 1, osteopontin, matrix metalloproteinases-2, and membrane-type 1 metalloproteinases gene expression in Mes1 cell, infected for a longer period (14 days). The results were confirmed by western blot analysis. Zymography analysis showed that *C. pneumoniae* modulated the *in-vitro* secretion of MMP-2 in Mes1 cells both at 7 and 14 days. Cell invasion, as measured by matrigel-coated filter, increased after 7 and 14 days infection with *C. pneumoniae*, compared with uninfected Mes1 cells. The results of this study suggest that *C. pneumoniae* infection might support cellular transformation, thus increasing lung cancer risk. [**Int Microbiol** 2014; 17(4):185-193]

Keywords: *Chlamydia pneumoniae* · cytotoxicity · human mesothelial cells · cellular transformation · tumoral markers

Introduction

Cancer is commonly defined as the uncontrolled growth of abnormal cells that have accumulated enough DNA damage to be freed from the normal restraints of the cell cycle. Although viral infections have been strongly associated with cancer [34,35], bacterial associations are also significant. Important mechanisms by which bacterial agents may induce carcinogenesis include chronic infection, immune evasion, and immune suppression. Several pathogenic bacteria, particularly those that can establish a persistent infection, can promote or initiate abnormal cell growth by evading the immune system or suppressing apoptosis [22]. In particular, some species or their toxins can alter host cell cycles or stimulate the production of inflammatory substances linked to DNA damage [7,37]. A separate discussion applies to intracellular pathogens that survive by evading the ability of the host to identify them as foreign.

Chlamydia pneumoniae is a Gram-negative bacillus and a compulsory intracellular parasite. *Chlamydia pneumoniae* infection is acquired during childhood, and the prevalence gradually increases to reach a maximum in middle age; it causes

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respiratory infection in more than 50% of adults, leading to a higher incidence of pneumonia, as well as bronchitis, sinusitis, rhinitis, and exacerbation of chronic obstructive pulmonary disease [12]. The route of transmission is usually by aerosol and, in most cases, these infections are mild. The resulting clinical course is acute symptomatic illness followed by chronic respiratory symptoms. After acute infection, *C. pneumoniae* intracellular life cycle is characterized by the development of metabolically inert (antibiotic-resistant) atypical "persistent" inclusions. Persistent infection is a permanent source of bacterial antigen, promoting chronic inflammation; *C. pneumoniae* infections are thought to induce a state of persistent inflammation [38]. It has been suggested that persistent *C. pneumoniae* inflammation would correlate with increased risk of lung cancer, by inducing chronic pulmonary inflammation [20,25]. In the complex framework of interactions between the infective agent and immune response, superoxide oxygen radicals, TNF-a, IL-1β and IL-8 play an essential role, contributing to lung tissue damage and DNA damage that eventually result in carcinogenesis [36].

The time between acquiring the infection and cancer development is most often years or even decades as seen in cancers associated with *Helicobacter pylori*, *Salmonella typhi*, and *Streptococcus bovis* infections. The association of *C. pneumoniae* infection with lung cancer risk has been variable [21]; this could reflect the retrospective nature of some studies, small sample sizes, or inadequate adjustment for confounding due to smoking [27]. In addition, modest reliability of serologic assays and the lack of a validated marker for chronic infection have precluded an exact estimation of the etiologic role of *C. pneumoniae* [27]. Further information on the role of *C. pneumoniae* in lung cancer could be provided by studies using additional markers of infection and inflammation. The implementation of molecular biomarkers in the early diagnosis of lung cancer has been a long standing goal. Particular focus was given in identifying such biomarkers in bronchial washings in individuals with a high risk of developing lung cancer [33].

Calretinin (CR) is a vitamin D-dependent calcium-binding protein involved in the physiological buffering of excess cytosolic calcium ions, calcium transport, and protection against calcium ion overload [42]. CR is expressed in a subpopulation of neurons in the central and peripheral nervous system and is consistently up-regulated in reactive mesothelial cells and in epithelioid malignant mesothelioma (MM). Until now CR has been mostly considered as a highly useful marker for the identification of MM and based on the fact that CR is not expressed, or it is undetectable, in normal mesothelial cells *in vivo*.

The Wilms' tumor gene 1 (*WT1*) is a major regulator of cell growth and development in the embryo kidney, adult urogenital system and central nervous system [41]. The transcription factor WT1 has been found activated also in some human neoplasias, including Wilms tumor, gastrointestinal and pancreatobiliary tumors, urinary and male genital tumors, breast and female genital tumors, brain tumors, soft tissue sarcoma, osteosarcoma, malignant melanoma [32], and in mesothelioma cells [2]. WT1 expression has been reported to be markedly low in cells of normal healthy individuals, with the exception of the CD34+ hematopoietic progenitors [18]. WT1 protein expression has been observed in endothelial cells during angiogenesis, thus it can be a useful marker to distinguish between vascular proliferations and vascular malformations.

Osteopontin (OPN) is a secreted phosphoglycoprotein that binds the integrin and CD44 families of receptors and plays a major role in tumorigenesis, tumor invasion, and metastasis [44]. Increasing data have shown that high expression levels of OPN are associated with invasion, progression, or metastasis in malignant tumors of the pancreatic cancer, gastric cancer, liver cancer, and lung cancer [43].

Mesothelioma progression depends on an interaction with mesothelial cells that provide membrane-type 1 metalloproteinases (MT1-MMP) necessary to activate pro-matrix metalloproteinases 2 (pro-MMP-2) to facilitate migration through an extracellular matrix layer. In particular, MT1-MMP predominantly converts pro-MMP-2 to the intermediate forms but not to mature MMP-1 form. MMP-2 has been reported as a characteristic for pleural malignant mesothelioma, and has been suggested as a predictive marker for poor prognosis [14].

On the basis of the results reported in the literature, we were prompted to investigate if chlamydial infection could contribute to *in vitro* cellular transformation by up-regulating the gene expression of three known biomarkers of the on-going neoplastic transformation, that is CR, WT1 and OPN. Here we report experimental evidence that sustained *C. pneumoniae* infection may cause cellular transformation as evaluated through the induced expression of CR, WT1 and OPN.

Materials and methods

Cell culture and treatments. Primary cultures of mesothelial cells (Mes1) were isolated and developed from pleural biopsy of a patient who was cytologically, histologically and immunohistochemically confirmed as having non-malignant pleural mesotheliomas [4]. Tissue specimens were minced and incubated in growth medium 1:1 composition of DMEM and Ham's F12 medium (Invitrogen) supplemented with 20% fetal calf serum (GIBCO BRL.

Grand Island, NY), penicillin (0.1 mg/ml), streptomycin (0.1 mg/ml), epidermal growth factor (10 μg/ml), insulin (5 mg/ml) and hydrocortisone (0.2 mg/ml). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 14 days to achieve 75% confluence. Mes1 cells displayed a highly flattened cellular morphology composed of tightly packed non-overlapping cells, which covered the entire surface of the culture dish following confluence. Mes1 was analyzed by RT-PCR for the expression of carcinoembryonic antigen (CEA, negative marker), WT1, mesothelin and calretinin.

Propagation of *Chlamydia pneumoniae*. *Chlamydia pneumoniae* (AR39) was propagated in HEp-2 cell monolayers as described by Roblin *et al* [39]. In brief, *C. pneumoniae* was inoculated onto a pre-formed monolayer of HEp-2 cells in 35-mm diameter wells, centrifuged at 1000 ×*g* at 25°C for 60 min and incubated at 37°C with 5% CO_2 for 1h. Supernatants were replaced with growth medium consisting of RPMI-1640 containing 1 μg/ml cycloheximide. Infected cultures were incubated at 37°C in 5% CO_2 for 3 days. *Chlamydia pneumoniae* was harvested by disrupting HEp-2 cells with glass beads followed by sonication and centrifugation at 250g to remove cellular debris. Supernatants containing *C. pneumoniae* were further centrifuged at 20,000*g* for 20 min to pellet elementary bodies (EB). The EB pellet was then suspended in sucrose–phosphate–glutamate buffer, aliquoted and stored at -70°C. Infectivity titers of chlamydial stocks were evaluated by the titration of the inclusion-forming units (IFU) per millilitre in HEp-2 cells. These titers were used to determine the infectious doses for the cell line studied. Cell cultures and chlamydial stocks were confirmed to be free of *Mycoplasma* infections using 4,6-diamidino-2-phenylindole fluorescent staining (Sigma-Aldrich S.r.l., Milan, Italy). In addition, contamination with *Mycoplasma* was excluded regularly by *Mycoplasma*-PCR using specific primers (MWG Biotech, Martinsried, Germany).

In vitro infection. Mes1 cells were seeded onto coverslips in 24-well plates at a density of 5 ×10^4 cells/well in the growth medium. The cells were then infected with *C. pneumoniae* by centrifugation at 1000*g* for 60 min at a multiplicity of infection (MOI) of 4 IFU/cell (a preliminary study showed this MOI to be the optimum rate) and incubated for 3 days. For some experiments, determinations were performed at 3 days post infection because of the complicated, biphasic developmental cycle lasting up to 3 days. The count of IFU chlamydial was evaluated as described by Salin et al. [40]. In brief, at indicated times, the medium was removed from the wells and the coverslips were washed twice with PBS and fixed in methanol for 10 min. The coverslips were allowed to dry and the chlamydial inclusions were stained with fluorescein isothiocyanate (FITC)-conjugated anti-MOMP monoclonal antibody (Dako Cytomation, Milan, Italy), according to manufacturer's instructions. The stained inclusions were examined under a fluorescence microscope (Axioskop 2, Carl Zeiss, Milan, Italy) at 400×. The number of the formed inclusions was counted from four eye fields of each coverslips and calculated using the following formula: [(inclusions in control – inclusions in treated sample) / inclusions in control] ×100.

Cell proliferation and cell viability. Cell viability was evaluated with methyltetrazolium (MTT), which determines the activity of cellular (mitochondrial) respiration, and can be considered as a metabolic rate of cells. Mes1 cultures were incubated with DMEM alone (negative control) or with *C. pneumoniae* (MOI = 4) at 37°C in 5% CO_2. The number of living cells was determined by colorimetric MTT assay (3-[4.5-dimethyl-2.5 thiazolyl]-2.5 diphenyltetrazolium bromide; Sigma–Aldrich S.r.l.) according to the procedure of Boonyanugomol, et al. [3] after 3, 7 and 14 days. The absorbance of a formazan product in the tissue culture media was measured at 650 nm using a microplate reader, and the results were expressed as the mean percentage of the control cells. MTT assay data were confirmed by counting infected and uninfected cells in a Bürker chamber. For viability evaluated by microscopy examination, the cells were observed at a magnification of 200× (CK 40 Olympus Microscope). The viability of the cells infected with *C. pneumoniae* and those of the controls were confirmed by the activity of the lactate dehydrogenase (LDH) released in the supernatants [10]. Briefly, 50 μl of the aliquots of cell supernatants were mixed with 25 μl of LDH reagent (Sigma-Aldrich S.r.l.) and incubated at room temperature for 30 min. The LDH activity was calculated by measuring the increase in absorbance at 490 nm and was expressed as a percentage of the control values.

Morphological analysis. To monitor whether Mes1 cells were capable of supporting the growth of *C. pneumoniae* in vitro, at 3 days post infection, the infected cells were fixed with 100% methanol and stained for the inclusion bodies using a fluorescein–isothiocyanate (FITC)-conjugated anti-MOMP monoclonal antibody (Dako Cytomation, Milan, Italy). Morphological features of Mes1 cells infected with *C. pneumoniae* were examined at a magnification of 400× by confocal fluorescence microscopy (Axioskop 2). Determinations were performed at MOI = 4 and after 3 days post infection because this multiplicity and time of infection had been found to be the best in preliminary experiments.

Real-time PCR analysis. Semi-confluent Mes1 cells (10^6/well) were infected with *C. pneumoniae* for 7 and 14 days. Total RNA was isolated with the High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy) from Mes1 cells, infected and non-infected with *C. pneumoniae*. Three hundred nanograms of total cellular RNA were reverse-transcribed (Expand Reverse Transcriptase, Roche Diagnostics) into complementary DNA (cDNA) using random hexamer primers (Random hexamers, Roche Diagnostics), at 42°C for 45 min according to the manufacturer's instructions. Real-time PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Diagnostics; LightCycler 2.0 Instrument) using 2 μl of cDNA, corresponding to 10 ng of total RNA in a 20 μl final volume, 3 mM $MgCl_2$ and 0.5 μM each primer (final concentration). Primer sequences and annealing temperatures are shown in Table 1. A melting curve was made at the end of each amplification to ensure the absence of non-specific reaction products. The accuracy of mRNA quantification depends on the linearity and efficiency of the PCR amplification. Both parameters were assessed using standard curves generated by increasing amounts of cDNA. Quantification was based on the threshold cycle values, measured in the early stage of the exponential phase of the reaction. All quantifications were normalized to the housekeeping gene β-actin. The percentage of gene expression increase was calculated using the following formula: [(gene expression in unstimulated conditions – target gene expression) / gene expression in unstimulated conditions] ×100.

Protein extraction and western blot analysis. Semi-confluent Mes1 cells (10^6/well) were infected with *C. pneumoniae* for 7 and 14 days. Cells were scraped with 1 ml PBS, and the cell pellet was homogenized with 300 μl ice-cold buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton, 1.5 mM $MgCl_2$, 5 mM EGTA) supplemented with 20 mM sodium pyrophosphate, 40 μg/ml aprotinin, 4 mM PMSF, 10 mM sodium orthovanadate, 25 mM NaF. Total extracts were cleared by centrifugation at 10,000 rpm for 30 min at 4°C and assayed for the protein content by Bradford's method. Fifty μg of protein from each cell lysate were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Filters were then stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. In addition, protein normalization was verified by densitometric analysis of bands. Blots were blocked overnight with 5% non-fat dry milk, then incubated with anti-MMP-2 (H-76) rabbit polyclonal antibody, OPN (sc-21742) mouse monoclonal, WT1 (sc-192) rabbit polyclonal, calretinin (sc-365956) mouse monoclonal and anti-tubulin mouse monoclonal antibody (Santa Cruz Biotechnology) 1:200 in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 8) for 2 h at room temperature. After washing with 0.1% Tween-20 PBS, the filters were incubated with 1:2500 peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins for 1 h at

Table 1. Human sense and antisense primer sequences. Sequences and conditions of the oligonucleotide primers used in real time-PCR analysis

Sense and antisense sequences	Conditions
5′-CATACTACGGATGTTTGACTT-3′ 5′-TCACGCTCTCTGAGTCTGG-3′	40 cycles at 95°C for 5s 56°C for 8s, 72°C for 17s
5′-CTCTTGTACGGTCGGCATCT-3′ 5′-CAGCTGGAGTTTGGTCATG-3′	40 cycles at 95°C for 5s 56°C for 8s, 72°C for 17s
5′-CACCTGTGCCATACCAGTTAAAC-3′ 5′-GGTGATGTCCTCGTCTGTAGCATC-3′	40 cycles at 94°C for 5s 53°C for 11s, 72°C for 21s
5′-TGACGGTAAGGACGGACTC-3′ 5′-TGGAAGCGGATTGGAAAC T-3′	40 cycles at 94°C for 5s 57°C for 7s, 72°C for 14s
5′-CTGGGCCATGCCCTGGGGCTC-3′ 5′-CAGGAACAGAAGGCCGGGAGG-3′	40 cycles at 94°C for 5s 64°C for 4s , 72°C for 8s
5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′	40 cycles at 95°C for 5s 64°C for 8s , 72°C for 20s

22°C. They were extensively washed and finally analyzed using the ECL system (Amersham).

Gelatin zymography. Semi-confluent Mes1 cells were plated in 6-well plates (35 mm diameter) and infected with *C. pneumoniae* for 7 and 14 days. Gelatinolytic activity of MMP-2 was determinated using the method of Heussen and Dowdle [13] adapted for minigel format. Conditioned media of each sample were centrifuged at 6000 rpm for 20 min and the protein content of the supernatant was estimated by Bradford's method. Twenty μg of sample were mixed with an equal volume of 2×non-reducing sample buffer and each sample was separated by 10% (w/v) polyacrylamide gel containing 2 mg/ml of gelatine (Sigma). After electrophoresis, the gel was incubated in 2.5% Triton X-100 for 1hour to remove SDS and then overnight at 37°C in the developing buffer (50mM Tris-HCl, pH 7.6, containing 0.2 M NaCl, 5mM $CaCl_2$ and 0.02% (w/v) Brij-35). The gel was stained for 45 min in 40% methanol/10% glacial acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R 250 and de-stained in the same solution without Coomassie Brilliant Blue.

Cell invasion assay. Cell invasion assays were carried out in Boyden chambers under serum-free conditions as previously described [1]. The 10-μm pore-size-polycarbonate filters were coated with 5 μg/ml fibronectin and then with Matrigel (BD Biosciences) 25 μg/ml. After 14 days *C. pneumoniae* infection, Mes1 cells were trypsinized and placed in the upper compartment of the Boyden chamber in serum-free medium, and FBS 10% was placed in the lower compartment as the chemoattractant. Cells were allowed to attach and spread at 37°C in 5% CO_2 for 24 h. The cells on the upper surface of the filter were completely removed by wiping with a cotton swab, while those that had traversed the Matrigel and attached to the lower surface of the filter were fixed in ethanol, stained with hematoxylin and counted in 10 random fields/filter at 200×. In parallel, the control cells were assessed for viability and counted with trypan blue. The number of cells that had invaded was normalized to analyze the effects on cell viability.

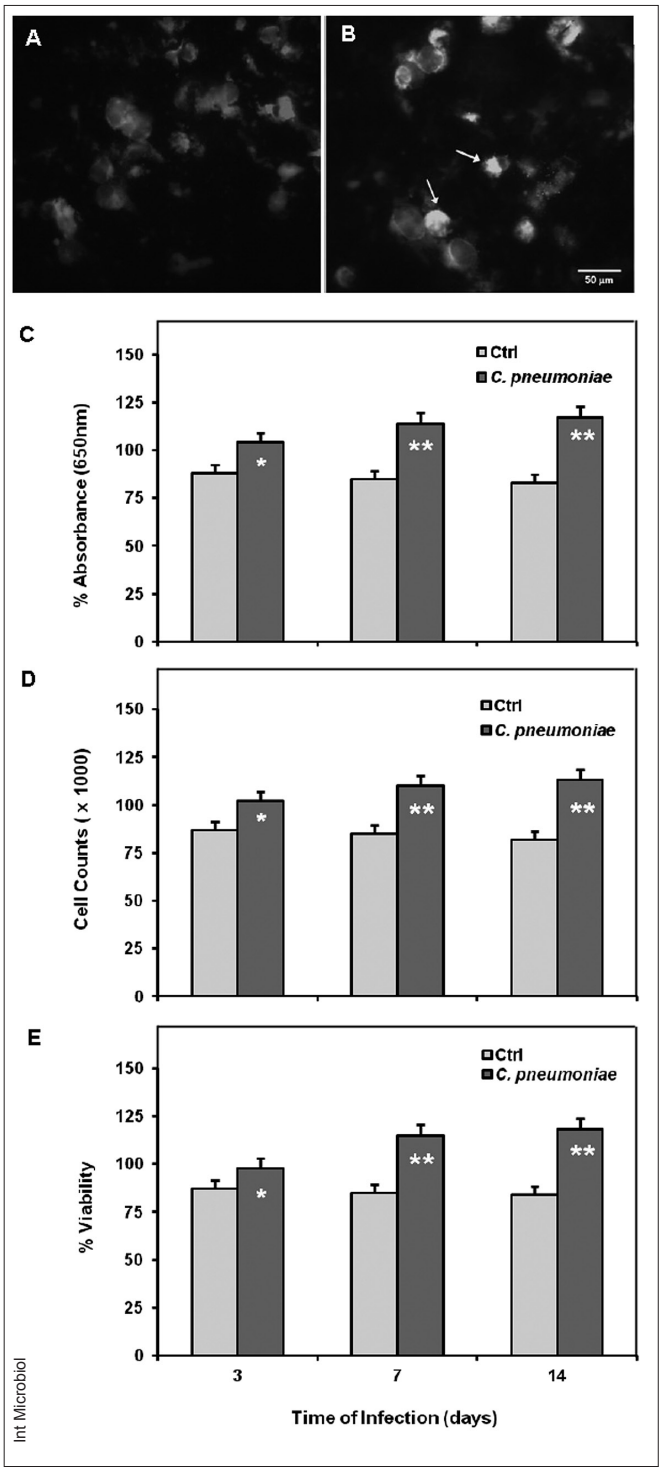
Statistical analysis. Each experiment was performed at least three times. The results are expressed as means ± standard deviations (SD). Student's *t* test was used to determine statistical differences between the means, and $P < 0.01$ was considered a significant difference.

Results

Mes1 cells proliferation and viability *Chlamydia pneumoniae*–infected. Confocal fluorescence microscopy using an anti-*Chlamydia* monoclonal antibody revealed that Mes1 cells had many intracellular inclusion bodies after 3 days of infection (Fig. 1B) compared with control cells (Fig. 1A), whereas at 1 and 2 days post infection the cells showed only few intracellular inclusion bodies (data not shown).

We examined the effect of *C. pneumoniae* infection on the proliferative activity of Mes1 cells, by both the colorimetric MTT and LDH assay. After 3 days exposure to *C. pneumoniae*, the cell number was slightly modified compared to test cultures and controls (Fig. 1C–E). After 7 days of incubation with *C. pneumoniae*, an increased proliferative activity was observed. In particular, during this period, the proliferative response of Mes1 cells incubated with *C. pneumoniae*, determined by colorimetric assay and cell counting, showed an increase of 34.1% and 29.4%, respectively, compared to the cells alone (Fig. 1C,D), while the viability, determined by LDH activity, increased by 35.2% compared to the control cells (Fig. 1E). After 14 days of incubation, cell proliferation and viability still increased reaching values of 40.9%, 37.8% and 40.4%, respectively.

Expression of CR, WT1 and OPN in infected *Chlamydia pneumoniae* Mes1 cells. To investigate whether chlamydial infection might promote cellular transfor-



mation we analysed *CR* and *WT1* gene expression, two known biomarkers of the on-going neoplastic transformation. As shown in Fig. 2A and C, Mes1 cell infected for 14 days showed a strong induction of *CR* and *WT1* gene expression. Shorter period of infection (7 days) induced only a slight modification of the biomarkers. On contrary, prolonged period of infection (21 days) were not investigated since Mes1 cells morphology was modified, showing the typical feature of cell suffering (data not show). To reinforce the result obtained we went to analyse osteopontin (*OPN*) gene expression, a marker reported to play an important role in tumorigenesis. Our results demonstrated that *OPN* was also up-regulated in infected Mes-1 cells (Fig. 2E), resulting in a major increase after 14 days *C. pneumoniae* infection. Finally, the results obtained on *CR*, *WT1* and *OPN* gene expression were all confirmed by western blot analysis (Fig. 2B, 2D and 2F).

CR and WT1 overexpression increases the in vitro invasive potential of Mes1 cells. In order to evaluate whether CR and WT1 overexpression might favour cell invasion we examined gelatinase *MMP-2* gene expression and its activator membrane type1-MMP (MT1-MMP). As shown in Fig. 3A and 3B, *MT1-MMP* and *MMP-2* gene expression were both induced after 7 and 14 days of Mes1 cells infection. However, the expression of these genes was stronger after 14 days infection, as it happened for the other markers analyzed. Also western blot analysis (Fig. 3C) showed that MMP-2 pro-enzyme and the mature 62k-Da enzyme of MMP-2 were more strongly increased after 14 days in Mes1 infected cells, compared with the point at 7 days and the control, suggesting the activation of the gelatinase. To confirm the result obtained we analyzed by zymography whether *C. pneumoniae* affects the *in-vitro* secretion of MMP-2 by Mes1 cells. As shown in Fig. 3D, MMP-2 secretion increased after 7 days infection, with a stronger increase after 14 days, compared with uninfected cells.

To strengthen the results we had obtained, we investigated the ability of *C. pneumoniae* to increase Mes1 cell invasion. Cell invasion, as measured using a modified Boyden chamber with a Matrigel-coated filter, was increased after 7 and 14

Fig. 1. Representative experiments of Mes1 cells infected with *Chlamydia pneumoniae*. (**A)** untreated Mes1 cells. (**B)** three days after incubation the infected cells were fixed with methanol and stained for the inclusion bodies using an anti-*Chlamydia* monoclonal antibody. Intracellular inclusion bodies (arrow). Images were collected using confocal fluorescence microscopy at ×400 magnification. (**C-E)** effect of Mes1 cells infected or uninfected (Ctrl) with *C. pneumoniae* (MOI = 4) on proliferation (**C**); cell counts (**D**); and cell viability (**E**). (**C**) Proliferation was determined by a colorimetric MTT assay (OD at 650 nm) after 3, 7 and 14 days of culture of infected and uninfected cells. Results are expressed as the mean percentage of control cells. Data are means ± SD of three different experiments. (**E**) Viability was determined by LDH activity and is expressed as percentage of control values after 3, 7 and 14 days culture of infected cells. Data are means ± SD of three independent experiments. The asterisk indicates a statistically significant difference between the experimental test and the control test. * $P < 0.05$ ** $P < 0.01$ *versus* Mes1 cells alone.

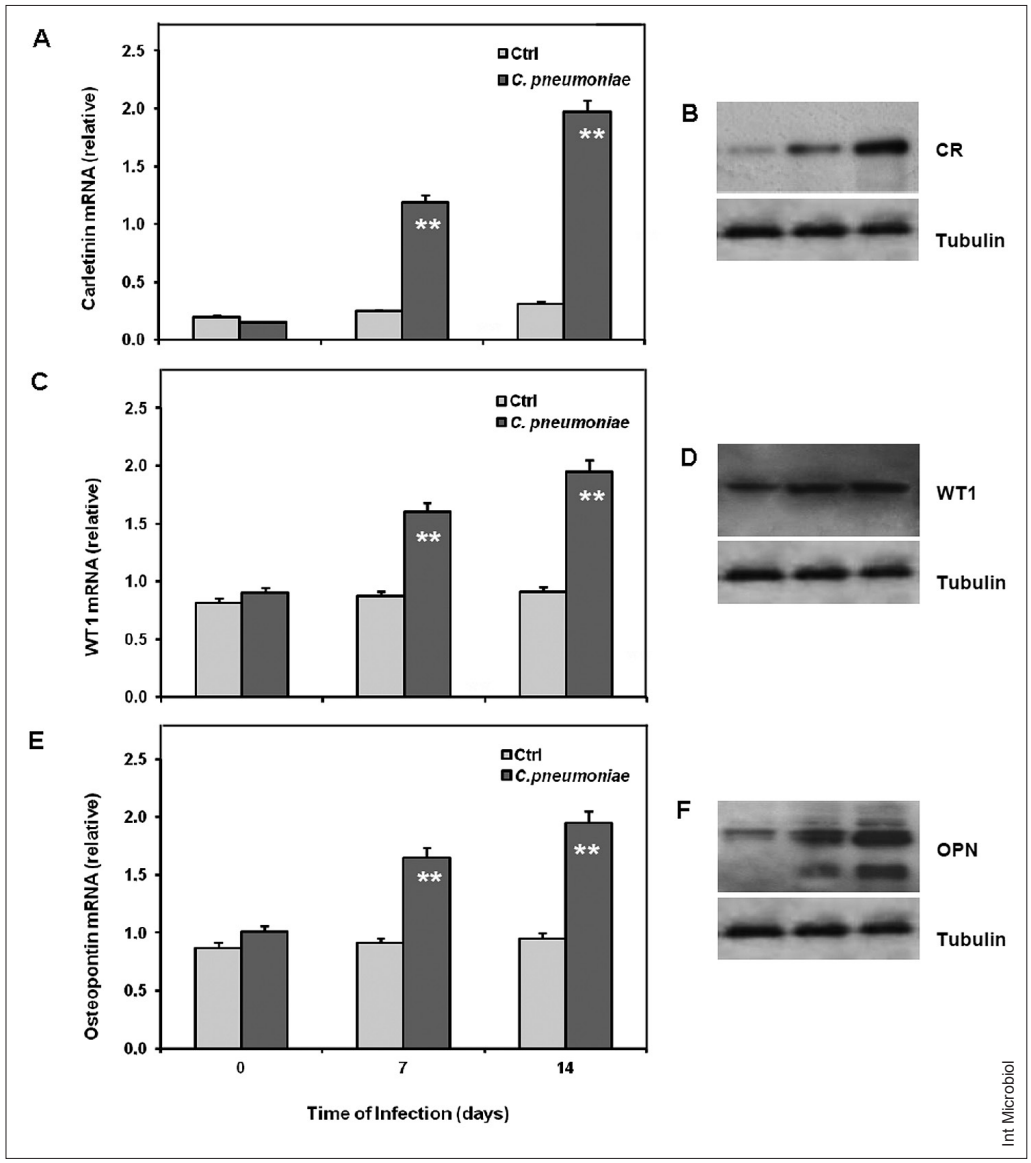


Fig. 2. Real time PCR analysis using specific primers for CR (**A**), WT1 (**C**) and OPN (**E**). Mes1, cells infected or not (Ctrl) with *Chlamydia pneumoniae* for 7, 14 days; Ctrl, untreated Mes1 cells. The columns are the mean values from three independent experiments with three duplicates. Significant differences compared to untreated cells are indicated as follows: ** $P < 0.01$. (**B**, **D** and **F)** western blot analysis of CR, WT1 and OPN in Mes1 cells after exposure to *C. pneumoniae*. Line 1, untreated cells; lines 2-3, Mes1 cells infected with *C. pneumoniae* for 7 and 14 days respectively.

days infection with *C. pneumonia* (26% and 60% respectively), compared with uninfected Mes1 cells (Fig. 4).

Discussion

Chlamydia pneumoniae infection has been suggested to be strongly associated with lung carcinoma. However, only seroepidemiological studies have indicated such a potential relation [20]. In fact, high *C. pneumoniae* antibody titers have been observed in lung cancer. Specifically, high IgA against *C. pneumoniae* were reported to be correlated with squamous cell carcinomas and to a lesser extent with small cell carcinomas and adenocarcinomas of the lung [28]. To our knowledge, up to now there is not any report about *C. pneumoniae* infection inducing transformation of human mesothelial cells.

In our study, Mes1 cells infected with *C. pneumoniae* showed many intracellular inclusion bodies, confirming that

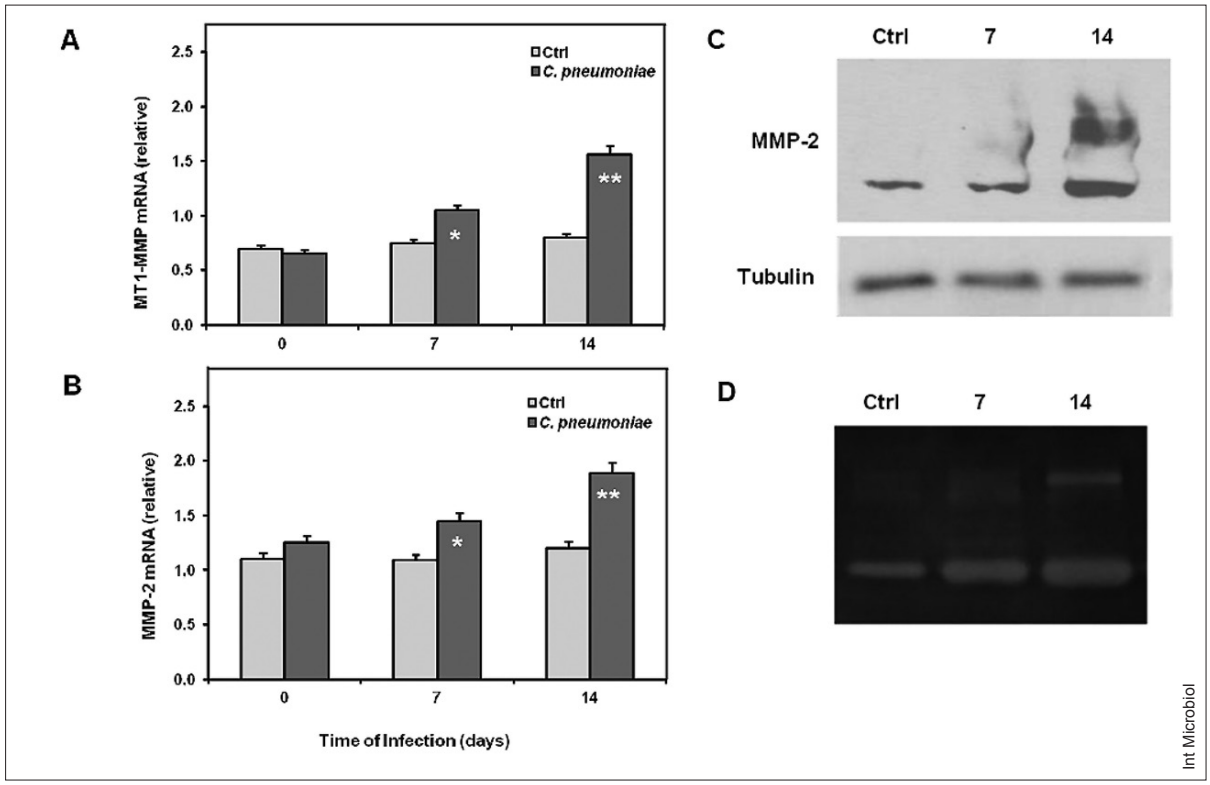


Fig. 3. Real time PCR analysis using specific primers for MT1-MMP (**A**) and MMP-2 (**B**). Mes1, cells infected or not (Ctrl) with *Chlamydia pneumoniae* for 7 and 14 days; Ctrl; untreated Mes1 cells. the columns are the mean values from three independent experiments with three duplicates. Significant differences compared to untreated cells are indicated as follows: * $P < 0.05$ and ** $P < 0.01$. (**C**): western blot analysis of MMP-2 in Mes1 cells after exposure to *C. pneumoniae*. Line 1, untreated cells; lines 2-3, Mes1 cells infected with *C. pneumoniae* for 7 and 14 days respectively. (**D**) MMP-2 secretion in Mes1 cells after exposure to *C. pneumoniae*. Line 1, untreated cells; lines 2-3, Mes1 cells infected with *C. pneumoniae* for 7 and 14 days respectively.

the microorganism was able to invade and replicate in this cellular type. In addition, an increased proliferative activity was demonstrated in *C. pneumoniae*-infected Mes1 cells. It is known that *C. pneumoniae* infection causes irregular apoptosis in tissues by unknown mechanisms [5]. Apoptosis and cellular proliferation have a pivotal role in carcinogenesis. Hyperproliferation simultaneously reduces the time available to repair mutations in DNA and also increases the risk of spontaneous mutation due to errors in DNA replication [29]. It has been reported that *C. pneumonia* infection of endothelial cells triggers both vascular smooth muscle cells proliferation and the mitogenic activity of platelet-derived growth factor. There is also evidence that *C. pneumoniae* infection in endothelial cells induces the production of different mediators of inflammation, among which MMP, which contributes to plaque destabilization [8]. Using molecular biomarkers for the early diagnosis of lung cancer has been a long standing objective. Particular focal point was given in identifying such biomarkers in bronchial washings in individuals with a high risk of developing lung cancer. The *WT1* gene was originally identified as a tumor suppressor gene, recently proposed to act as a chameleon gene in malignancies, i.e. functioning also as an oncogene [17]. It is expressed in a small number of human tissues [31] and in various cancer cells [32]. The marker has been usually considered to be positive in mesothelioma. Calretinin is one of the first markers that have proven to be useful in the diagnosis of malignant mesothelioma [9], it being positive in 80–100% of malignant mesotheliomas [23,30]. Mesothelin and calretinin are proteins strongly expressed in mesothelial cells and mesotheliomas, whereas WT1 is mainly expressed in mesothelioma cells [11]. The expression of mesothelin and calretinin in our primary cell culture confirmed the mesothelial differentiation (data not shown). Our results showed a strong induction of CR and WT1 gene expression in Mes1-infected cells , thus confirming the ability of *C. pneumoniae* to induce cellular transformation. To further support our results, we analysed another marker of tumor progression, OPN, and found a significant up-regulation of *OPN* gene ex-

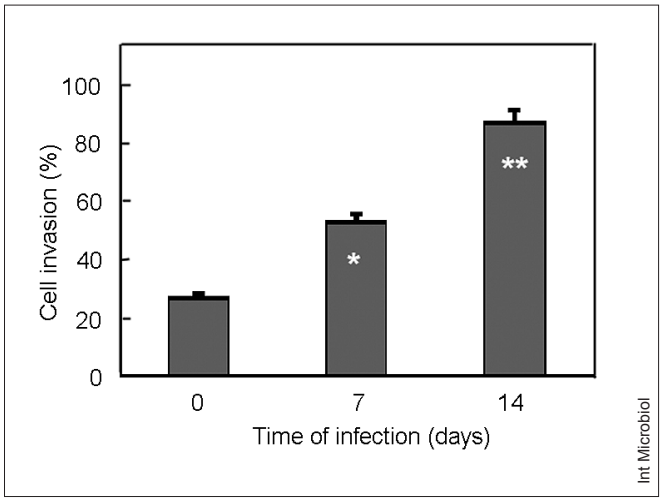


Fig. 4. Inhibitory effect of *Chlamydia pneumoniae* infection on Mes1 invasion. Control and *C. pneumoniae*-infected Mes1 cells were plated onto a Matrigel modified Boyden chamber. Cells were allowed to attach and spread for 24 h. Only cells that had passed through the Matrigel were stained and counted. The average number of cells per field is expressed as a percentage of the control after normalizing for cell number. The results are the mean values of three different experiments. Significant differences compared to untreated cells are indicated as follows: * $P < 0.05$ and ** $P < 0.01$.

pression in infected Mes1 cells. This result is of interest since OPN has been shown to bind and/or activate pro-matrix metalloproteinase-3 (pro-MMP-3) and pro-MMP-9, and to activate phosphatidylinositol 3-kinase (PI3K)/protein kinase B pathway, promoting cell migration and cell survival [16]. Thus, OPN might contribute to sustained cell proliferation by contrasting apoptotic cell death and the elimination of acquired mutations. MMP-2 and its activator membrane type1-MMP (MT1-MMP) are molecules known to be linked to aggressive tumor progression, poor survival, and high risk for metastasis [14,46]. Interestingly, our results demonstrated that MMP-2 and MT1-MMP gene expression were both induced after 14 days of Mes1 cells infection. The results were confirmed measuring the enzymatic activity by zymography. Finally, we found out that *C. pneumoniae* influenced the invasive behavior of Mes1 cells.

The results here reported indicate that *C. pneumoniae* infection might support cell transformation. Epidemiological data in the literature support the idea that *C. pneumoniae* infection might trigger lung carcinoma. Laurila, et al. [24] reported that *C. pneumoniae* infection was present principally in patients with small-cell and squamous cell carcinomas, among 230 smokers with lung carcinoma. According to some studies, smoking assists *C. pneumoniae* to invade the lung [20]. *Chlamydia* infection is believed to increase lung cancer risk by inducing chronic pulmonary inflammation. Inflammatory mediators, while offering protection by destroying invading pathogens, can inhibit apoptosis and enhance cell proliferation, both of which can promote mutation and carcinogenesis [19,26]. Another study suggests that chronic inflammation could be responsible for the observed link between *Helicobacter pylori* and carcinogenesis [15]. Similarly, *C. pneumoniae* infection might represent a risk factor aggravating the condition, in particular, of some classes of workers exposed to asbestos fibres at high risk of developing mesothelioma. Note that many of the bacterial infections that support oncogenesis are often asymptomatic. When the pathways toward malignancy start and when they become irreversible, though, are aspects not yet fully understood [6]. Two other intracellular bacteria, *Mycoplasma fermentans* and *M. penetrans*, phylogenetically close relatives of Chlamydiae, have been reported to transform C3H mouse embryo cells *in vitro* by a multistage progression characterized by C-myc mRNA over-expression [45].

Because lung carcinoma and mesothelioma usually carry a dismal prognosis, there is urgent need to develop early diagnostic markers and effective therapies against chronic *C. pneumoniae* infections. To our knowledge, this is the firs report of *C. pneumoniae* infection inducing trasformation of human mesothelial cells, which supports the idea that *C. pneumoniae* infection might increase the risk of lung carcinoma. Further information on the role of *C. pneumoniae* in lung cancer could be provided by studies using additional markers of infection and inflammation.

Competing interests. None declared.

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