Carcinogenesis vol.25 no.8 pp.1477–1484, 2004
doi:10.1093/carcin/bgh091

Carcinogenic properties of proteins with pro-inflammatory activity from *Streptococcus infantarius* (formerly *S.bovis*)

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Several studies reported linkage between bacterial infections and carcinogenesis. *Streptococcus bovis* was traditionally considered as a lower grade pathogen frequently involved in bacteremia and endocarditis. This bacterium became important in human health as it was shown that 25–80% of patients who presented a *S.bovis* bacteremia had also a colorectal tumor. Moreover, in previous experiments, we demonstrated that *S.bovis* or *S.bovis* wall extracted antigens (WEA) were able to promote carcinogenesis in rats. The aim of the present study was: (i) to identify the *S.bovis* proteins responsible for *in vitro* pro-inflammatory properties; (ii) to purify them; (iii) to examine their ability to stimulate *in vitro* IL-8 and COX-2 expression by human colon cancer cells; and (iv) to assess *in vivo* their pro-carcinogenic potential in a rat model of colon carcinogenesis. The purified S300 fraction, as determined by proteomic analysis, contained 72 protein spots in two-dimensional gel electrophoresis representing 12 different proteins able to trigger human epithelial colonic Caco-2 cells and rat colonic mucosa to release CXC chemokines (human IL-8 or rat CINC/GRO) and prostaglandins E_2, correlated with an *in vitro* over-expression of COX-2. Moreover, these proteins were highly effective in the promotion of pre-neoplastic lesions in azoxymethane-treated rats. In the presence of these proteins, Caco-2 cells exhibited enhanced phosphorylation of the three classes of MAP kinases. Our results show a relationship between the pro-inflammatory potential of *S.bovis* proteins and their pro-carcinogenic properties, confirming the linkage between inflammation and colon carcinogenesis. These data support the hypothesis that colonic bacteria can contribute to cancer development particularly in chronic infection/inflammation diseases where bacterial components may interfere with cell function.

Introduction

A marked resurgence of interest in the gastrointestinal commensal flora and in local host–microbe interaction has been observed since it was recognized that intestinal bacteria could be implicated in the pathogenesis of several inflammatory diseases like Crohn's disease or ulcerative colitis. Both diseases are commonly presumed to result from altered host responses to normal intestinal bacterial flora (1), and are associated with cancer risk (2,3). In addition, several studies have related bacterial infections to carcinogenesis. For example, *Helicobacter pylori* is well known to be associated with gastric cancer in human (4), *Citrobacter rodentium* with mucosal colonic hyperplasia in mice (5,6) and *Streptococcus bovis* with colorectal cancer (7).

The hallmark of *H.pylori* infection is the development of chronic inflammation in the gastric mucosa (8) and it seems that chronic infection and subsequent chronic inflammation are responsible for the maintenance and development of pre-existing neoplastic lesions (9). *In vivo*, gastric infection with *H.pylori* induced local mucosal production of various cytokines including IL-1β, TNF-α, IL-6 and IL-8, which are implicated in the intestinal inflammatory process (10,11). On the other hand, neutrophils are able to release reactive oxygen species or reactive nitrogen species, which can cause DNA damage *in vivo* (12).

Epidemiological studies indicated that non-steroidal anti-inflammatory drugs (NSAIDs) decreased the relative risk of gastrointestinal carcinomas (13,14). The main target of NSAIDs is cyclooxygenase (COX), especially COX-2, which is involved in inflammation and is induced by mitogens, hormones, growth factors and cytokines like IL-8. It was shown that COX-2 is over-expressed in up to 85% of colorectal adenocarcinomas (15). COX-2, through the production of prostaglandins, promotes either cellular proliferation (16,17), angiogenesis (18) or inhibits apoptosis (19). All these functions confer to the inflammatory response a promoter effect on the carcinogenic process.

Streptococcus bovis, occasionally present in the human gastrointestinal tract flora, is considered as a lower grade pathogen frequently involved in bacteremia and endocarditis. However, it was shown in various studies that 25–80% of patients with an *S.bovis* bacteremia exhibited also a colorectal tumor (7,20–24). On the other hand, it was reported that fecal carriage of *S.bovis* was increased in patients with colon carcinoma (25). Taken together these data suggest a correlation between *S.bovis* and the development of colorectal cancer. The mechanism underlying this association is not known. Nevertheless, we showed in a previous study that in rats pretreated with a chemical carcinogen (azoxymethane), *S.bovis* acted as a promoting factor leading to the formation of pre-neoplastic lesions in the colon (26). We also found: (i) that *S.bovis* enhanced the expression of proliferation markers like PCNA (proliferative cell nuclear antigen) and polyamines; and (ii) that *S.bovis* bacterial wall extracted antigens (WEA) had the same effects as the whole bacteria. We also observed a strong CINC/GRO release in rats treated with *S.bovis* or *S.bovis* WEA indicating colonic mucosal inflammation.

Abbreviations: AOM, azoxymethane; COX-2, cyclooxygenase-2; ERK 1/2, extracellular signal-regulated kinases; JNK, c Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; PGE_2, prostaglandins E_2; WEA, wall extracted antigens.

The aim of the present study was: (i) to purify the *S.bovis* proteins responsible for the pro-inflammatory properties of WEA; (ii) to examine the ability of these proteins to modulate the expression of IL-8 and COX-2 in human colonic epithelial Caco-2 cells; (iii) to determine the impact of mitogen-activated protein kinases (MAPKs) phosphorylation on the release of these mediators; (iv) to assess *in vivo* their pro-carcinogenic potential in a rat model; and (v) to characterize them by a proteomic analysis.

Materials and methods

Bacterial strain and antigen preparation

The bacterial strain used in this study was *S.bovis* NCTC 8133, recently reclassified as *Streptococcus infantarius* subsp. *infantarius* (27). Bacteria were cultured at 37°C in brain–heart infusion broth (Difco Laboratories, Detroit, MI) with 1% glucose under anaerobic conditions. The WEA were prepared from 18 h culture as already described (28). WEA was re-suspended in buffer A (Bis Tris buffer 20 mM, pH 7) and kept frozen at −20°C until use.

Fractionation of WEA

Streptococcus bovis WEA were first fractionated by anion-exchange chromatography using a Waters HPLC system equipped with a MonoQ® HR 5/5 column (Pharmacia, Saint-Quentin-en-Yvelne, France). The column was equilibrated with buffer A and WEA fraction (15 mg) was loaded onto the column. Elution was performed at a flow rate of 1 ml/min with a gradient of NaCl (0.05–1 M) in buffer A. The absorbance of the eluate was monitored at 280 nm, 2 ml fractions were collected and tested for their ability to induce IL-8 release in Caco-2 cells. The active fractions were pooled, dialyzed against buffer A, concentrated and submitted to gel-filtration chromatography on a Sephacryl™ S300 column (100 × 2.5 cm; Pharmacia), pre-equilibrated with buffer A supplemented with NaCl 50 mM. Fractionation was conducted at a flow rate of 0.2 ml/min; 2 ml fractions were collected and tested for their ability to induce IL-8 release. The active fractions were pooled, dialyzed against buffer A, concentrated (2 mg proteins/ml) and stored at −80°C until use as S300 fraction.

Cell culture

Cells of the human colonic epithelial cell line Caco-2 (ECACC 86010202), originally derived from an adenocarcinoma (29,30), were used between passages 10 and 30. Caco-2 cells were grown to confluence, at 37°C in a 95% air and 5% CO_2 humidified atmosphere, in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 0.1 mM sodium pyruvate, 1% non-essential amino acids, penicillin–streptomycin (100 IU/100 μg/ml) and 10% heat-inactivated fetal calf serum (FCS) as described previously (31). Cell monolayers were treated with trypsin–EDTA (0.25 g/l and 2 mM, respectively) and 200 μl of the cell suspension (4×10^3 cells) were added to each well of 96-well microtiter plates (Costar, Dutscher Brumath France). Cells were allowed to grow to confluence for 7 days and to differentiate for 4–5 days more, and finally were grown for 24 h in serum-depleted medium. Cell viability was examined by the MTT test (32).

Activation of cells and pro-inflammatory mediators assay

Differentiated Caco-2 cells (6×10^5 cells/well) were stimulated with 200 μl of either *S.bovis* WEA (50 μg/ml) in serum-free DMEM supplemented with antibiotics, or single or pooled fractions eluted from the chromatography columns. After 20 h incubation, supernatants were removed and used to determine IL-8 and prostaglandins E_2 (PGE_2) levels. For inhibition assays, cells were pre-incubated for 1 h at 37°C with 100 μl of either PD98059 (100 μM) or SB203580 (1 and 20 μM) diluted in serum-free DMEM supplemented with antibiotics.

Western blot detection of COX-2 and MAPKs

Differentiated Caco-2 cells (1.0×10^6 cells) in suspension were incubated for various times with 200 μl of serum-free medium supplemented with antibiotics containing either WEA or S300 active fraction (100 μg/ml), or anisomycin (10 μg/ml) for positive control of c Jun-N-terminal kinase (JNK) and p38 kinase activation. After stimulation, the cells were centrifuged (300 *g*, 10 min, 4°C), and the pellets were suspended for 20 min in 100 μl of ice-cold lysis buffer [1% Triton X-100, 20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF and 1× protease inhibitor cocktail (Complete™ Mini EDTA-free, Roche Diagnostics, Meylan, France)]. The Triton X-100-soluble proteins were separated by centrifugation (13 000 *g* for 10 min at 4°C) and the supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electro-transferred to PVDF membranes. Membranes were blocked using 5% milk in TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl) for 1 h at 25°C. The blots were then incubated with either rabbit anti-COX-2 (Cayman Chemical, Ann Arbor, MI), or rabbit anti-phosphorylated extracellular signal-regulated kinases (ERK) 1/2, JNK and p38 (Promega, Charbonnière, France) polyclonal antibodies or mouse anti-total p38 monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY) in TBS-Tween (0.05% Tween 20) for 20 h at 4°C, followed by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (2 h at 25°C). The blots were detected by enhanced chemiluminescence according to the manufacturer's instructions (ECL™ Western blotting detection reagents, Amersham Pharmacia Biotech, Orsay, France).

Animals and treatments

The experiments were conducted according to the National Research Council Guide for use and care of laboratory animals with the authorization (no. 00573) of the French ministry of agriculture. Male Wistar rats ($n = 24$) weighing 230–245 g, were housed under standardized conditions (22°C; 60% relative humidity; 12/12 h light/dark cycle; 20 air changes/h) with free access to controlled isocaloric diet (234 kcal/kg/day; UAR A05) and tap water. All animals received i.p. injections of azoxymethane (AOM), 15 mg/kg body wt once each week for 2 weeks. One week after the last injection with AOM (week 3), the rats were randomly divided into three experimental and one control groups of six rats each.

The rats were inoculated with 1 ml of either *S.bovis* WEA (100 μg, group I), or S300 fraction (6 μg, group II) diluted in PBS twice a week for 5 weeks via intragastric gavage. In order to give the same quantity of proteins to all animals, PBS was supplemented up to 120 μg proteins with porcine skin gelatin in each group. The control group consisted of AOM-treated rats and received 120 μg of porcine skin gelatin in 1 ml PBS. One day after the last gavage (week 7), the animals were killed.

Assessment of aberrant crypts and tumors in the colon

The determination of aberrant hyperproliferative crypts and tumors was performed on a segment of 5 cm in length corresponding to the distal part of the colon. The segment was washed with physiological saline, cut open, pinned out flat and fixed in 4% buffered formalin. The colon was stained with 0.2% methylene blue for 5 min, rinsed in Krebs–Ringer buffer, placed onto a glass slide and examined microscopically using a low power objective (×5) for assessment of the number of aberrant crypts (33,34), aberrant crypt foci (ACF) and the presence of tumors. All counts were made by two blinded observers (F.G. and M.S.-G.). The criteria for the identification of aberrant crypts were: (i) an increased size; (ii) a thicker epithelial cell lining; and (iii) an increased pericryptal zone relative to normal crypts.

Determination of pro-inflammatory mediators in rat mucosa

Colonic mucosa samples (80–160 mg) were homogenized in 1 ml of PBS containing EDTA (10 mM), PMSF (2 mM) and gentamicin (40 μg/ml) and the suspensions were centrifuged (10 000 *g*, 15 min, 4°C). The supernatants were used to determine their content in CINC/GRO and PGE_2 and the results are expressed as nanograms of CINC/GRO or PGE_2 per milligram of proteins contained in the colonic mucosa supernatant.

Two-dimesional electrophoresis and in-gel digestion and mass spectrometry analysis

A Genomic Solution Investigator two-dimensional (2-D) electrophoresis system (Genomic Solutions, Ann Arbor, MI) running Tris–Tricine chemistry (35) was used to separate the S300 fraction. The proteins (180 μg) were focused through the first dimension over a pH range of 4–7, prior to application on a second-dimension SDS-8–20% gradient polyacrylamide gel. Gel was stained with Coomassie blue. Separated gel spots were subjected to tryptic digestion and were digested overnight at room temperature in 25 mM ammonium bicarbonate buffer. The resulting peptides were extracted from the gel and analyzed by nanoscale capillary liquid chromatography-tandem mass spectrometry (nanoLC-MS-MS). Purification and analysis were performed on a C18 capillary column (Pepmap, LC Packings) using a CapLC capillary LC system (Waters, Milford, USA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF II, Micromass, Manchester, UK) (36). Elution was performed with 30% acetonitrile–1% aqueous trifluoroacetic acid.

Mass data acquisitions were piloted by MassLynx software using automatic switching between MS and MS-MS modes. The internal parameters of Q-TOF II were set as follows. The electrospray capillary voltage was set to 3.0 kV, the cone voltage set to 30 V and the source temperature set to 80°C. The MS survey scan was *m/z* 300–1500 with a scan time of 1 s and a interscan time of 0.1 s. When the intensity of a peak rose above a threshold of eight counts,

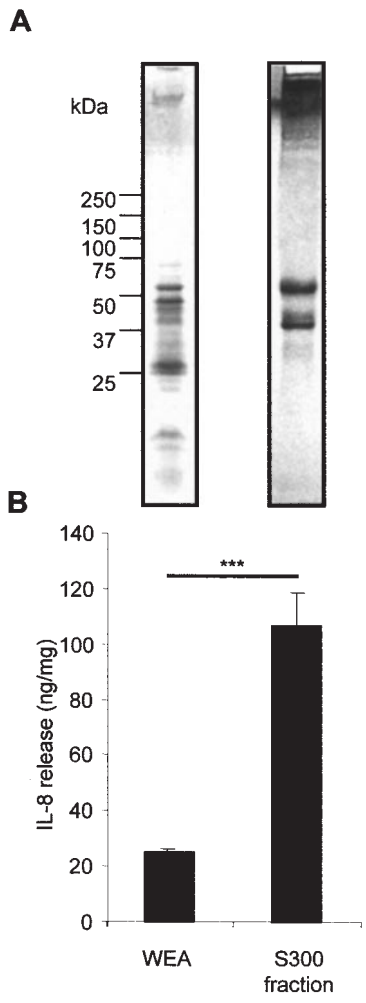

Fig. 1. IL-8-inducing proteins from *S.bovis* WEA. 10% SDS–PAGE profile of WEA and S300 fractions stained with Coomassie blue (**A**). IL-8-inducing activity of WEA and S300 fraction (**B**). Caco-2 cells were stimulated for 20 h at 37°C with each fraction or medium alone (control). The results are expressed as specific activity and are mean values ± SE for triplicate determination from three different experiments. The results are statistically significant: $^{***}P < 0.001$.

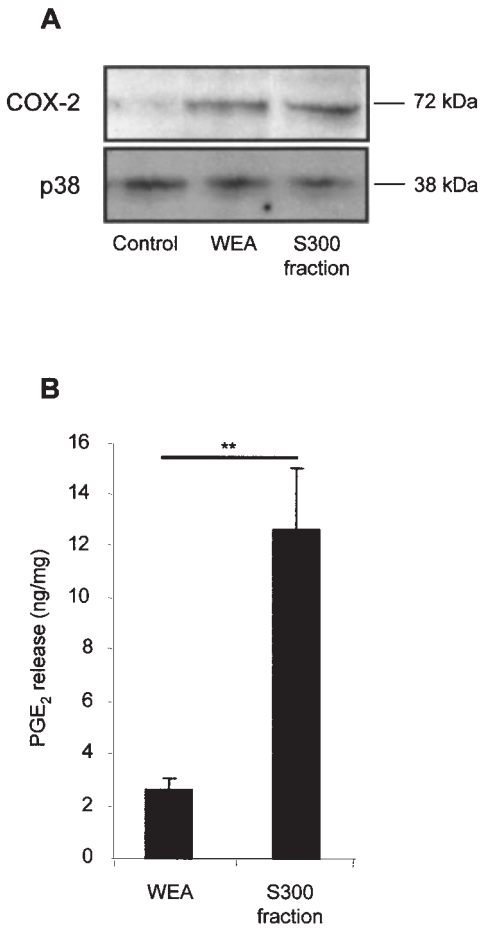

Fig. 2. COX-2 expression and PGE_2 release by IL-8-inducing fractions. Caco-2 cells were incubated in medium alone (control) or treated with WEA or S300 fraction. Cell lysates were analyzed by western blotting with anti-COX-2 polyclonal antibodies. Loading controls have been revealed with anti-total p38 monoclonal antibodies (**A**). PGE_2-inducing activity of WEA and S300 fraction (**B**). The results are expressed as specific activity and are mean values ± SE for triplicate determination from three different experiments. The results are statistically significant: $^{**}P < 0.01$.

tandem mass spectra were acquired. Normalized collision energies for peptide fragmentation were set using the charge-state recognition files for +1, +2 and +3 peptides ions. The scan range for MS-MS acquisition was from *m*/*z* 50 to 1500 with a scan time of 3 s and an interscan time of 0.1 s. Fragmentation was performed using argon as the collision gas and with a collision energy profile optimized for various mass ranges of precursor ions.

Mass data collected during a nanoLC-MS-MS analysis were processed and converted into a PKL file to be submitted to the search software MASCOT (Matrix Science, London, UK). Searches were done with a tolerance on mass measurement of 0.25 Da in MS mode and 0.5 Da in MS/MS mode.

Analytical procedures

Protein content was evaluated according to Bradford method and protein profile was analyzed by 10% SDS–PAGE under reducing conditions. The enzyme immunoassay kits for PGE_2 (Cayman Chemical) and for human IL-8 (cross-reacting with rat CINC/GRO; R&D systems, Abingdon, UK) were used according to the manufacturer's instructions.

Statistics

Data are reported as means ± SE. Statistical difference between groups were evaluated by one-way ANOVA analysis and specific differences were identified using the Student–Newman–Keuls multiple comparison test.

Results

Isolation of IL-8-inducing fractions

WEA (15 mg) was first fractionated by anion-exchange chromatography on a MonoQ HR5/5 column eluted with a linear gradient of 0.05–1 M NaCl and tested for IL-8-inducing activity. The major fractions with IL-8-inducing activity, eluted between 200 and 350 mM NaCl, were pooled (9.6 mg) and fractionated by gel-filtration chromatography on a Sephacryl S300 column, and tested for IL-8-inducing activity. The active fractions eluted in the column void volume were pooled and termed S300 fraction (0.9 mg). SDS–PAGE analysis shows that the S300 fraction exibits around nine bands (Figure 1A) after staining with Coomassie blue as compared with the numerous bands present in WEA.

Stimulation of Caco-2 cells with WEA and S300 fraction for 20 h resulted in the reproducible production of IL-8 (Figure 1B). The S300 fraction displayed a 106.7 ± 12.1 ng IL-8-specific activity while WEA displayed a 24.9 ± 1.5 ng IL-8-specific activity. These results show that one or more component(s) of WEA with IL-8 inducing activity are present in the S300 fraction.

COX-2 expression and PGE_2 release by IL-8-inducing fractions

Since over-expression of COX-2 has been reported in the majority of colorectal adenocarcinoma, we then examined the action of WEA and S300 fraction on COX-2 expression in Caco-2 cells by western blotting. Activation of cells with WEA resulted in a significant increase in COX-2 expression within 6 h (Figure 2A), which decreases rapidly at 15 and 24 h (data not shown). Similarly, S300 fraction led to an increased expression of COX-2 within a 6 h stimulation time of Caco-2 cells (Figure 2A) as compared with the control, which is correlated with a 5-fold increase in PGE_2 levels in comparison with WEA (Figure 2B). These results clearly show that *S.bovis* IL-8-inducing components are also able to induce an over-expression of COX-2 by Caco-2 cells, which is correlated with increased release of PGE_2.

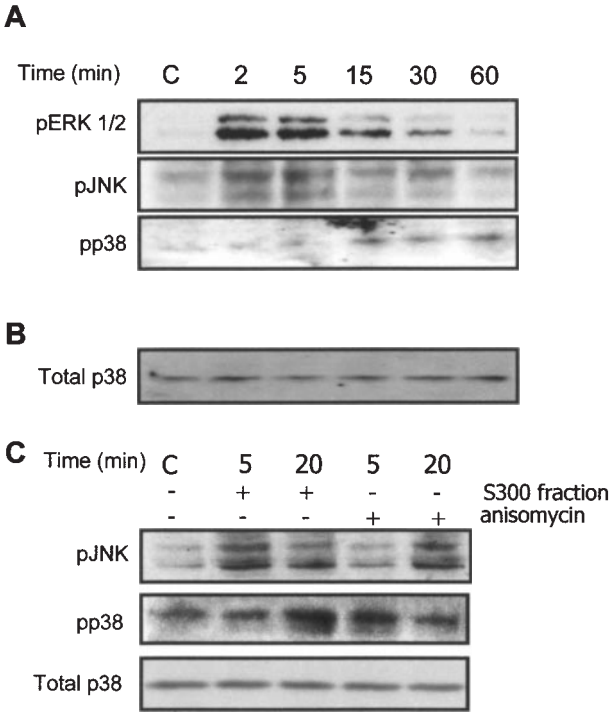

Fig. 3. Phosphorylation of ERK 1/2, JNK and p38 induced by S300 fraction in Caco-2 cells. Cells were stimulated with S300 fraction or anisomycin as positive control, for the indicated time at 37°C. Cell lysates were analyzed by western blotting with anti-phosphorylated ERK 1/2, JNK and p38 polyclonal antibodies (**A** and **C**). The membranes were stripped and reprobed with anti-total p38 antibodies (**B**). Controls were incubated with medium for 15 min. The results are representative of three different experiments.

IL-8-inducing S300 fraction activates MAPKs

As MAPKs appear to be required for IL-8 and PGE_2 induction (37,38) we examined the activation of ERK 1/2, JNK and p38 MAPKs in this process by detecting their phosphorylated forms. Western blot analysis (Figure 3A) shows that stimulation of human Caco-2 cells with S300 fraction resulted in an increased amount of phosphorylated ERK 1/2 and JNK, which was detectable within 2 min and lasted for 30 min. Phosphorylation of p38 was detectable only after 15 min and remained elevated for at least 60 min. These results show clearly that the S300 fraction stimulates the phosphorylation of the three classes of MAPKs. To further confirm that ERK 1/2, JNK and p38 MAPKs phosphorylation are involved in IL-8 and PGE_2 release, we used PD98059, a specific inhibitor of MEK1 and MEK 2 that blocks the ERK 1/2 signaling cascade, and SB203580, a selective inhibitor of p38 MAPK at low concentration and of both p38 and JNK MAPKs at high concentration. Both PD98059 and SB203580 inhibited IL-8 and PGE_2 release by Caco-2 cells (Figure 4). An inhibition of 82 and 93% of IL-8 and PGE_2 release, respectively, was obtained with PD98059. SB203580 at 1 μM inhibited 54 and 86% of IL-8 and PGE2 release, respectively, while at 20 μM, the respective inhibition reached 70 and 93%.

These results show that the phosphorylation of the three classes of MAPKs is necessary for the release of IL-8 and PGE_2 by Caco-2 cells stimulated by S300 fraction.

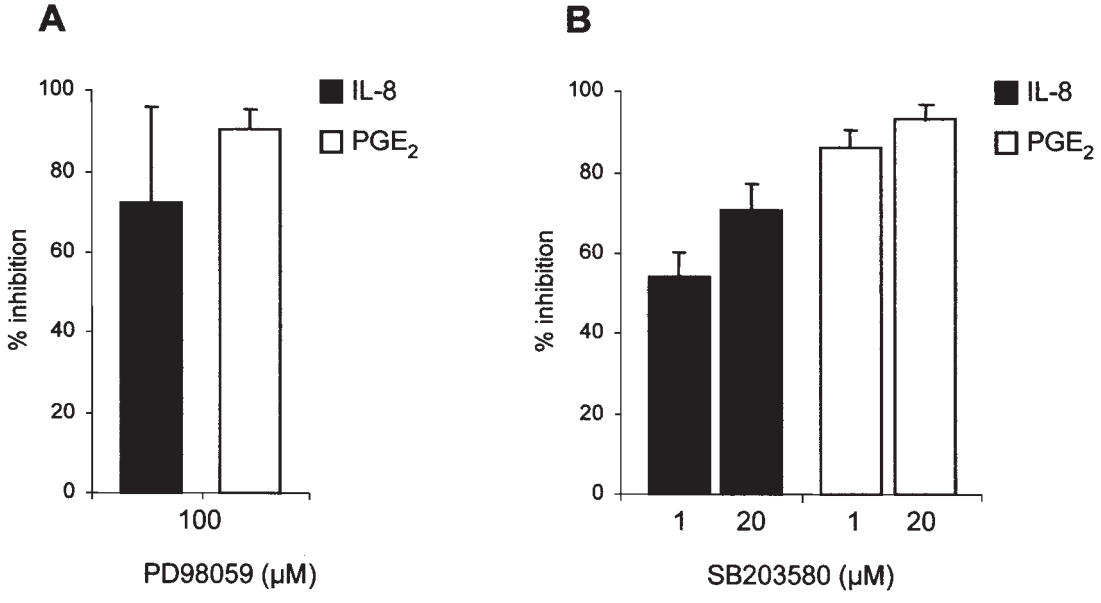

Fig. 4. PD98059 and SB203580 mediated inhibition of S300 fraction-induced IL-8 (filled square) and PGE_2 (open square) release by Caco-2 cells. Cells were pre-incubated (1 h at 37°C) with PD98059 (100 μM) or SB203580 (1 or 20 μM) and then stimulated for 20 h at 37°C with S300 fraction (100 μg/ml). The results are expressed as percentage inhibition of IL-8 and PGE_2 release and are means ± SE for triplicate determination from three different experiments.

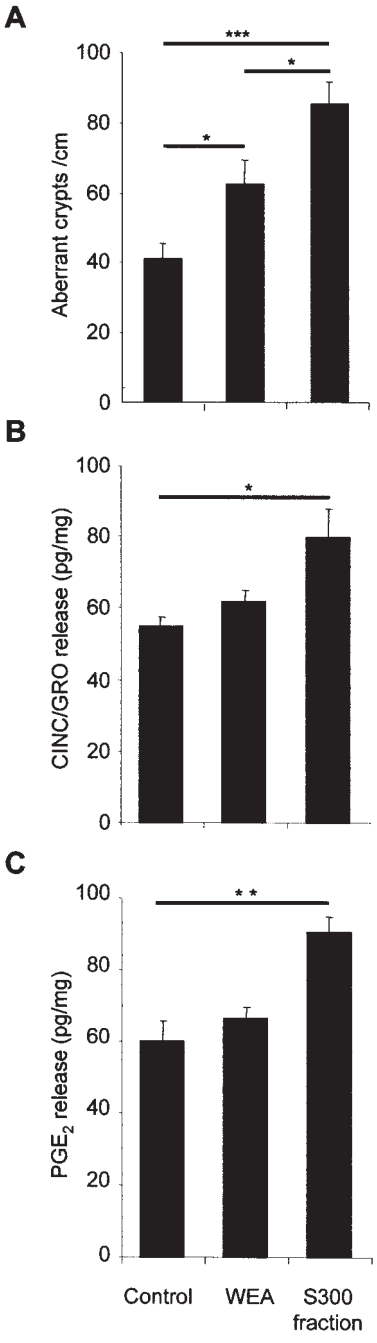

Fig. 5. Promotion in AOM-treated rats of aberrant crypts in the distal colon (5 cm in length) (**A**), and of CINC/GRO (**B**) and PGE_2 release (**C**) from mucosa samples by pro-inflammatory fractions. Rats were treated with AOM (15 mg/kg) and were then inoculated with either WEA or S300 fraction or PBS (control). The results are mean values ± SE of 6 animals/group. The results are statistically significant: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Animal experiments

The fact that the previously observed pro-carcinogenic effects of *S.bovis* WEA components were associated with increased production of CINC/GRO led us to test the ability of pro-inflammatory S300 fraction to induce the formation of ACF in AOM-pre-treated rats. At the end of the experiment, the body weight of the animals showed no significant differences and was 348–449 g in each group. As shown in Figure 5A, all rats injected with AOM developed abnormal and hyperplasic colonic crypts, regardless of the treatment. However, the administration of either WEA (group I) or S300 fraction (group II) resulted in a 1.5- and 2.1-fold increase of the number of aberrant crypts, respectively, when compared with control rats receiving AOM alone. Furthermore, only one small polyp was observed in group I, while five polyps were detected in group II (3/6 rats) and no polyp was observed in the AOM-controls.

CINC/GRO and PGE_2 were measured in mucosal samples of groups I and II. As shown in Figure 5B, CINC/GRO was detected in the mucosa of all rats. However, the level of CINC/GRO increased by 12 and 44% in rats administered with WEA and S300 fractions, respectively, in comparison with AOM controls. Although the colonic level of PGE_2 was not significantly enhanced in group I in comparison with the control group (10.7%), the amount of PGE_2 was increased by 51% in group II as compared with the control group (Figure 5C) suggesting a more advanced inflammatory state in the mucosa of animals treated with the S300 fraction.

Taken together, our results show that the *in vitro* IL-8 and PGE_2-inducing proteins are also pro-inflammatory and pro-carcinogenic mediators *in vivo* and this at a dose as low as 6 μg of proteins/rat.

MS analysis of S300 fraction

Proteins from the S300 fraction separated by 2-D gel electrophoresis revealed 72 Coomassie blue stained spots (Figure 6). The proteins from the 72 spots were subjected to in-gel tryptic digestion prior to nanoLC-MS-MS analysis. Sequence information for protein characterization was obtained via sequence homology searching in MASCOT database. However, as *S.bovis* proteins are poorly represented in the databases, 67 of the 72 spots were identified as 12 proteins having homologous function in related streptococcal species (Table I). These proteins can be shared among major cellular functions: proteins implicated in the biosynthesis of amino acids like NADP-specific glutamate dehydrogenase, stress proteins like GroEL, a heat shock protein, or superoxide dismutase or peroxide resistance protein that are expressed under stress conditions; proteins of importance for energy metabolism like phosphoglucose isomerase, aldolase, enolase or L-lactate dehydrogenase and proteins interfering with protein degradation like dipeptidase, cystein aminopeptidase C or glutamyl aminopeptidase.

The majority of the proteins detected showed isoelectric heterogeneity (charged isoforms), which is due to post-translational modifications such as phosphorylation, glycosylation or acylation. These proteins were identified under various masses except two proteins, namely L-lactate dehydrogenase (61,63,64) and enolase (59,61,62,65,66), which were only detected in spots with lower relative molecular mass than that usually ascribed suggesting some degradation.

Discussion

In the present work we have isolated some of the *S.bovis* cell-associated proteins released by the bacteria, which are able to stimulate intestinal cells to produce inflammatory mediators such as IL-8 and PGE_2 along with their ability: (i) to induce inflammation and (ii) to promote the progression of preformed pre-neoplastic lesions in colonic mucosa of rats. The partially purified fraction obtained after anion-exchange chromatography

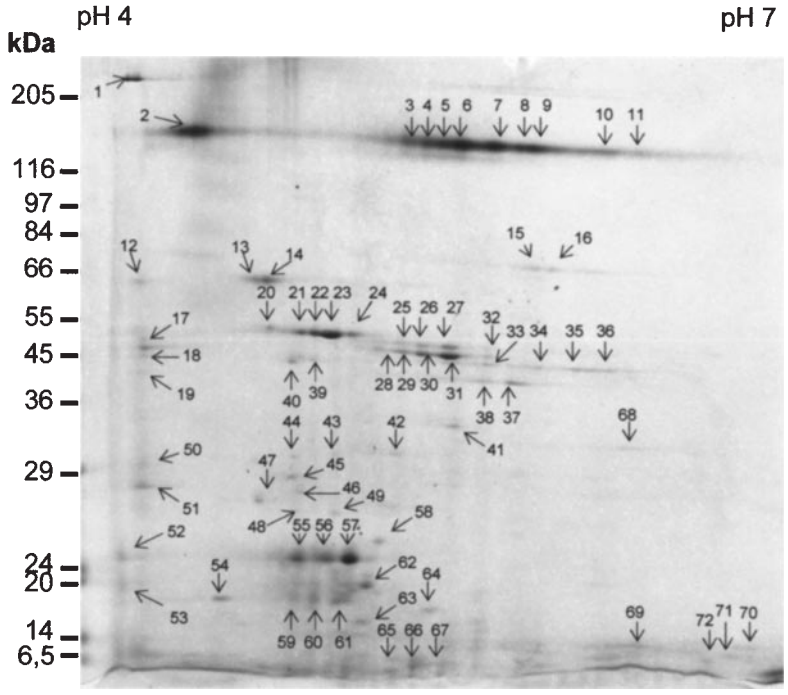

Fig. 6. Representation of the S300 fraction proteins separated by 2-D gel electrophoresis. Samples (180 μg) of protein were analyzed through the first dimension over a pH range of 4–7 prior to application on a second dimension SDS-8–20% gradient polyacrylamide gel. The gel was stained with Coomassie blue.

Table I. Identification by sequence homology of S300 fraction proteins categorized into putative functional groups

Function	Identification	Species
Amino acid biosynthesis		
Putative NADP specific glutamate dehydrogenase (17–19,21–38,43,47, 50,51,68,72)	Q8DUL2	*Streptococcus mutans*
Heat shock proteins and other stress proteins		
GroEL (48,55,59,60)	Q8KJ08	*S.bovis*
Superoxide dismutase (48)	Q8G8G2	*S.infantarius* subsp. *infantarius*
Peroxide resistance protein (Dpr) (2–11,42,56,57,61,67,72)	Q9KWH3	*S.mutans*
Energy metabolism		
Phosphoglucose isomerase (20–23,39,40,43–45,46,49,58)	Q8VVB7	*Streptococcus thermophilus*
Aldolase class II (42,43,61,65, 66,69–72)	Q9FA98	*S.bovis*
L-Lactate dehydrogenase (61,63,64)	Q59828	*S.bovis*
Enolase (59,61,62,65,66)	Q8E6G0	*Streptococcus agalactiae*
Protein degradation		
Putative dipeptidase (12–14,20,39)	Q8K848	*S.pyogenes*
Dipeptidase (1,13,14,20,54)	Q8E129	*S.agalactiae*
Glutamyl aminopeptidase (37,38)	Q8E225	*S.agalactiae*
Putative cysteine aminopeptidase C (41,42,49,50)	Q99YLO	*S.pyogenes*

and gel-filtration exhibited 12 different proteins as determined by proteomic analysis. These proteins are able to trigger epithelial Caco-2 cells and rat colonic mucosa to release CXC chemokines, potent leukocyte chemo-attractant and activating agents, and could therefore contribute to the establishment of a chronic bowel inflammation. The resulting activation of leukocytes may lead to the release of various other inflammatory mediators (NO, free radicals, peroxynitriles, etc.) (12), which could interfere directly or indirectly with the cell proliferation process. Similarly, Sharma *et al*. (39) found that infection with *cag+ H.pylori* strains induced IL-8 release, which correlates with the heightened inflammatory response observed in these patients.

Next, we found that the *S.bovis* proteins induce a 5-fold increase in PGE_2 secretion from stimulated Caco-2 cells as compared with cells stimulated with WEA. Such an enhanced production of PGE_2 was also measured in the intestinal mucosa of rats treated with these bacterial proteins. Subsequently, we demonstrated that PGE_2 release in Caco-2 cells is correlated with an over-expression of COX-2. For instance, Romano *et al*. (40) showed that *H.pylori* strains expressing some genes encoded by the *cag* pathogenicity island activate several host genes including these of COX-2, which is known to be associated with carcinogenesis. Over-expression of COX-2 in colon cancer and reduction in incidence of colorectal cancer in individuals taking NSAIDs led to the hypothesis that the products of COX-2 activity, namely prostaglandins, are significant contributors to carcinogenesis (41,42). COX-2 activation has a pivotal role in mucosal inflammation (43) and COX-2 activity is associated with inhibition of apoptosis (44) and enhancement of angiogenesis (45), both effects, which are favorable to cancer initiation and development.

Furthermore, the 12 *S.bovis* proteins exhibited a high effect on the promotion of pre-neoplastic lesions in the mucosa of AOM-treated rats. Our results could be paralleled to those of Newman *et al*. (6) who observed that *C.rodentium* infection of mice is characterized by colonic epithelial cell hyperproliferation, which promotes the development of colonic abnormal crypts containing many dividing cells.

We further investigated the molecular mechanisms by which IL-8 and COX-2 are regulated upon exposure of Caco-2 cells to *S.bovis* proteins and we found that stimulation of cells

resulted in the phosphorylation of all the three classes of MAPKs. Moreover, inhibition of MAPKs with specific inhibitors, leading to an inhibition of IL-8 and PGE_2 release, confirms the fact that MAPKs signaling pathways play an essential role in the induction of IL-8 and COX-2 genes. Several reports showed that MAPKs activation stimulates cells to undergo DNA synthesis and proliferation. Therefore, *S.bovis* proteins, like other bacterial components (46–48), could promote cell proliferation by triggering MAPKs, which might increase the incidence of cell transformation and the rate of genetic mutations. Furthermore, MAPKs and particularly P38 MAPK up-regulate the expression of COX-2, which is an important actor in tumorigenesis (49).

These data confirm the fact that the 12 *S.bovis* proteins could be related to the numerous microbial 'pathogens associated molecular patterns' which, after binding to their cognate 'pattern recognition receptors' on host cells, could interfere with numerous signaling pathways to induce an inflammatory response, cell proliferation and cell transformation. Some of the proteins identified are involved in cellular functions, however it has recently become apparent that a number of streptococcal proteins thought previously to be confined to the cytosol are also associated with the cell-surface or secreted into the external milieu (50). It has been suggested that unexpected proteins with an outer surface location may indicate secondary function for these proteins. For example, the phosphoglucose isomerase, described previously as glycolytic enzyme could act, outside the cell, as neuroleukin, autocrine motility factor and differentiation mediator (51). In the same way, stress protein HSP60 has been identified as the main *H.pylori* factor inducing IL-6 release from murine macrophages, and serum IL-6 concentrations have been linked to the status of *H.pylori*-induced gastric cancer (52).

Our data support the hypothesis that bacteria can contribute to cancer development, particularly in chronic infection/inflammation where normal cells can come under the influence of bacterial components, which interfere with regulatory systems cell proliferation and induce dysfunction of several metabolic and signaling pathways. Therefore, our model could serve as an excellent model of bacteria/inflammation associated sporadic cancer and may provide tools to understand the molecular mechanisms implicated in this process.

Acknowledgements

J.B. was the recipient of a grant from the French Ministry of Research and Technologies and I.S.N. of a grant from CAMPLP. This work was supported in part by La Ligue contre le Cancer, Comités du Bas-Rhin et du Haut-Rhin (67, 68, France).

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Received September 25, 2003; revised December 22, 2003; accepted January 14, 2004