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C-REACTIVE PROTEIN INHIBITS CELL MIGRATION AND ENHANCES EXPRESSION OF E-CADHERIN IN MURINE SQUAMOUS CELL CARCINOMA CELLS

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Abstract

C-reactive protein (CRP) is widely used as a biomarker of inflammation, though its functions are not fully understood. In the present study, we hypothesized that CRP reduces cancer cell invasion and migration by enhancing the expression of E-cadherin. NR-S1M cells, a murine squamous cell carcinoma line, were incubated for 48 h or 72 h with or without 10 or 50 μg/ml recombinant mouse CRP. Subsequent counts of viable cells showed that CRP had no significant effect on NR-S1M cell proliferation, nor did it affect invasion by the cells across a basement membrane matrix in Boyden chamber assays. By contrast, wound healing assays showed that CRP significantly weakened the migration potential of the cells. Moreover, Western and immunohistochemical analyses showed that CRP induced a corresponding increase in E-cadherin expression. These findings suggest that CRP may inhibit cancer cell migration, and thus metastasis, by strengthening cell-cell adhesion through increased E-cadherin expression.

Key words : C-reactive protein, E-cadherin, migration, epithelial-mesenchymal transition

Introduction

C-reactive protein (CRP) was first identified over 70 years ago and, although its function is still not fully understood, it is now being widely used as a biomarker of acute and chronic inflammation[1]. There have also been a number of reports showing an association between serum and tumoral CRP levels and cancer progression[2,3]. Furthermore, we recently observed that esophageal and lung cancer patients carrying the CRP 1846T/T genotype, which is associated with lower serum CRP levels, are more than 3 times likely to show lymph node metastasis[4,5].

Generally, cancer-related deaths are related to the metastatic spread of cancer cells from the original tumor to distant sites such as the lung or liver. Consequently, invasion and metastasis are considered the most important events to occur during tumor progression. In this regard, the role of cell-cell adhesion during tumorigenesis, invasion and metastasis has been the focus of numerous studies in cancer biology. E-cadherin is one of a major cell adhesion molecule, which evidence suggests may function as a tumor and invasion suppressor[6,7]. To further clarify the mechanism by which host CRP suppresses cancer cell metastasis, we examined the effect of CRP on E-cadherin expression and cell migration.

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Materials and Methods

Cell lines and culture

NR-S1M cells, a murine squamous cell carcinoma (SCC) cell line, were used. NR-S1M cells arose spontaneously in the C3H/He mouse strain and strongly metastasized to the lymph nodes[8]. We cultured NR-S1M cells in RPMI-1640 (Sigma-Aldrich, Saint Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS ; GIBCO, Grand Island, NY) and antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin, 250 μg/ml amphotericin B ; GIBCO) in a humidified incubator at 37°C under an atmosphere of 5% CO_2/95% air.

Cell proliferation assay

NR-S1M cells were plated in a 96-well plate to a density of 1×10^3 cells/well and incubated for 24 h in 100 μl of RPMI-1640 supplemented with 10% FBS. After removing the supernatants and washing the cells three times with PBS, they were incubated for 48 h or 72 h in 100 μl of RPMI-1640 containing 2.5% FBS without (control) or with 10 μg/ml or 50 μg/ml recombinant mouse CRP (r-CRP, R&D Systems, Minneapolis, MN). Following the incubation, the cells were washed, and the numbers of viable cells were determined using a Cell Titer-Glo Luminescent Cell Viability Assay kit as instructed by the manufacturer (Promega, Madison, WI). Viability was estimated based on the luminescent signal recorded using a microplate reader. Each cell was plated in triplicate in each experiment, and each experiment was repeated two times.

Invasion assay

Cells (1×10^6 cells/ml) were suspended in 300 μl of serum-free RPMI-1640 and loaded onto the inserts of Boyden invasion chambers coated with a uniform layer of dried basement membrane matrix solution (CytoSelect Cell Invasion Assay Kit ; Cell Biolabs, San Diego, CA). The lower wells were filled with 500 μl of RPMI-1640 without (control) or with 10 μg/ml or 50 μg/ml r-CRP. After 48 h, the invading cells were lysed and labeled with CyQuant GR fluorescent dye, after which the numbers of invading cells was estimated based on the fluorescence at 520 nm recorded using a fluorescence plate reader. Each cell was plated in triplicate in each experiment, and each experiment was repeated two times.

Wound healing assay

For wound healing assays (CytoSelect Wound Healing Assay ; Cell Biolabs), 500 μl of cell suspension (1×10^6 cells/ml) were added to the lower wells of invasion chambers and incubated overnight. The inserts and medium were then removed, and the cells were washed with medium. Thereafter, the divided cells were incubated in 500 μl of RPMI-1640 without (control) or with 10 μg/ml or 50 μg/ml r-CRP. Digital images of wound areas were captured after 0 h, 18 h and 36 h, and wound areas (percentage of each picture's total areas) were calculated using ImageJ software (NIH : National Institutes of Health, Bethesda, MD). Each cell was plated in duplicate in each experiment, and each experiment was repeated three times.

Western blotting

NR-S1M cells were plated in 60×15 mm dishes to a density of 5×10^5 cells/dish, after which they were incubated for 72 h in 3 ml of RPMI-1640 without (control) or with 10 μg/ml or 50 μg/ml r-CRP. After incubation, the supernatants were collected and centrifuged, and the collected cells were lysed in RIPA buffer (Thermo scientific, Sunnyvale, CA) containing a protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (Thermo Scientific). The protein content of the lysate was measured using a standard BCA assay, after which 10-μg aliquots of protein were subjected to 12.5% SDS-PAGE followed by transfer to PVDF membranes (Atto, Tokyo, Japan). The membranes were first blocked for 60 min in 3% skim milk in tris-buffered saline containing 0.1% Tween 20 (TBS-T) and then probed by incubation for 60 min with rabbit anti-E-cadherin antibody (1 : 500 dilution, sc-7870 ; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-α-smooth muscle actin (α-SMA) antibody (1 : 1,000 dilution, ab5694 ; Abcam, Cambridge, UK). The probed membranes were then incubated for 60 min with goat anti-rabbit IgG-HRP (1 : 5,000 dilution, sc-2004, Santa Cruz Biotechnology) and exposed to enhanced chemiluminescence reagents (sc-2048, Santa Cruz Biotechnology). This was followed by radiographic exposure and

development. At every step in the process, the membranes were washed three times for 5 min each in TBS-T, and all reactions were run at room temperature. In addition, digital images of all bands were captured, and the relative intensities of E-cadherin (ratios of E-cadherin/α-SMA) were calculated using ImageJ software (NIH : National Institutes of Health).

Immunofluorescence

NR-S1M cells were cultured on slides to a density of 5×10^5 cells/slide and then incubated for 48 h in 200 μl of RPMI-1640 alone (control) or with 10 μg/ml or 50 μg/ml r-CRP. The cells were then fixed for 15 min in 4% paraformaldehyde and permeabilized for 10 min using 0.1% Triton-X in PBS. The permeabilized cells were incubated first for 60 min in 1% blocking reagent and then for 60 min each with anti-E-cadherin (1 : 50 dilution, sc-7870 ; Santa Cruz Biotechnology) followed by Alexa 488-conjugated goat anti-rabbit IgG (1 : 200 dilution, Invitrogen, Karlsruhe, Germany). Finally, the slides were mounted using Prolong Gold Antifade Reagent (Invitrogen) and examined for E-cadherin using a microscope equipped with a 40× objective. At every step in the process, the slides were washed three times for 5 min each in PBS, and all reactions were run at room temperature.

Statistical analysis

Statistical analysis was performed using JMP 9 (SAS Institute, Cary, NC). Values are expressed as means± standard deviation. To assess quantitative differences between groups, analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparison tests were performed. Values of $P<0.05$ were considered significant.

Results

Effect of CRP on NR-S1M cell proliferation and invasion

To determine whether CRP influences cancer cell growth and invasion, we examined its effects on NR-S1M cells. Although cells incubated for 48 h or 72 h in the presence of 10 μg/ml or 50 μg/ml CRP exhibited a small reduction in proliferation, no significant change was observed ($P=0.0633$ at 48 h and $P=0.4680$ at 72 h) (Fig. 1). Similarly, incubation for 48 h with CRP had no significant effect on the magnitude of cell invasion ($P=0.1705$) (Fig. 2).

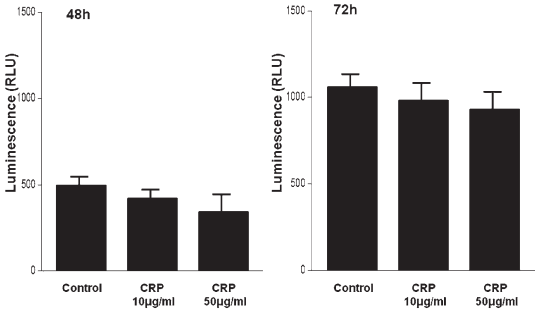

Fig. 1. Proliferation of NR-S1M cells was measured after culture for 48 h or 72 h in the presence of 10 μg/ml or 50 μg/ml CRP. All results are expressed as means±S.D. ($n=6$). No significant differences in cell proliferation were seen.

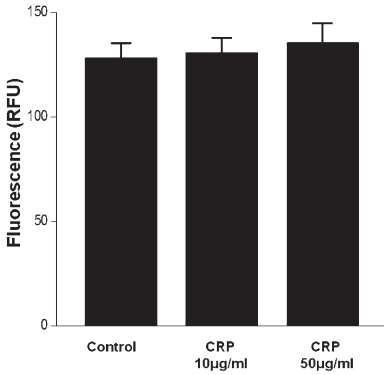

Fig. 2. Invasion by NR-S1M cells was measured after incubating the cells for 48 h in the presence of 10 μg/ml or 50 μg/ml CRP. There were no significant differences in the invasiveness of the cells. All results are expressed as means±S.D. ($n=6$).

CRP inhibits migratory potential

We next examined the cells' migration potential by carrying out a set of wound healing assays (Fig. 3A). We found that 18 h after wounding, the relative size of the wound area was significantly larger in NR-S1M cells treated with CRP (Fig. 3B, left panel). Moreover, the inhibitory effect of CRP on wound healing was even more

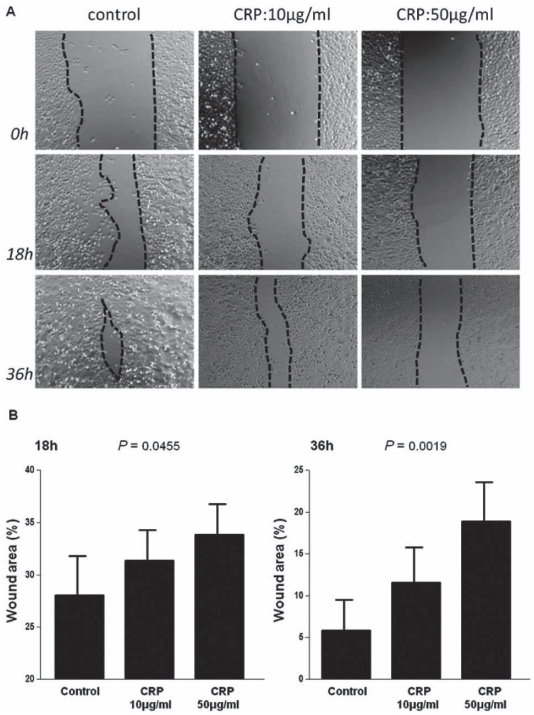

Fig. 3. (A) Representative wound healing assays showing the migration potential NR-S1M cells incubated for the indicated times with the indicated concentrations of CRP: magnification ×100. (B) After 18 h (left panel) and 36 h (right panel), wound areas were significantly larger in NR-S1M cell cultures treated with CRP than in control cultures. All results are expressed as means±S.D. (n=6).

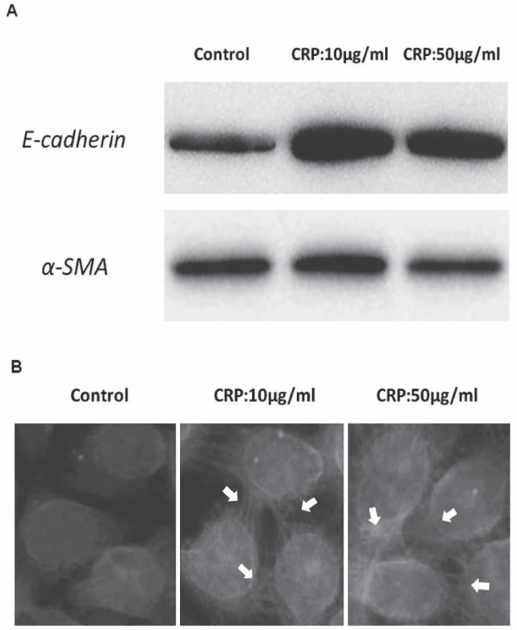

Fig. 4. (A) Western blots of NR-S1M cell lysate showing that 10 μg/ml and 50 μg/ml CRP enhances E-cadherin expression (135 kDa), as compared to control. By contrast, there was no effect on α-SMA expression (42 kDa). The ratios of E-cadherin/α-SMA were 0.76 in control, 1.28 in 10 μg/ml of CRP and 1.51 in 50 μg/ml of CRP. (B) Immunofluorescent images showing that CRP enhances expression of E-cadherin (arrows): magnification ×400.

apparent after 36 h (Fig. 3B, right panel). Thus CRP appears to dose-dependently inhibit the migration of NR-S1M cancer cells.

CRP increases E-cadherin expression

The inhibitory effect of CRP on NR-S1M cell migration prompted us to examine its effect on E-cadherin expression. Western blot analysis of NR-S1M cell lysates showed that whereas CRP had no effect on α-SMA expression, it clearly increased levels of E-cadherin expression (Fig. 4A). The relative intensities of E-cadherin (ratios of E-cadherin/α-SMA) were 0.76 in control, 1.28 in 10 μg/ml of CRP and 1.51 in 50 μg/ml of CRP, respectively. These finding were confirmed by immunofluorescent images, which showed that E-cadherin was strongly expressed in cells treated with CRP (Fig. 4B).

Discussion

Our findings demonstrate that treating cancer cells with CRP both enhances their expression of E-cadherin and reduces their migration. This suggests CRP may strengthen cell-cell adhesion through E-cadherin, which would in turn suppress cell migration.

For cancer cells to move from the primitive neoplastic tissue of a tumor into the surrounding host tissue they must acquire the ability to migrate[9]. That process involves the conversion of the tumor cells' phenotype from epithelial to mesenchymal (epithelial-mesenchymal transition (EMT))[10] and is closely associated with the acquisition of metastatic potential[11]. Notably, the principal

molecular feature of EMT is the downregulation of the cell adhesion molecule E-cadherin, which is present in the plasma membrane of most normal epithelial cells. E-cadherin functions as a tumor suppressor, inhibiting both invasion and metastasis[12], and its downregulation frequently occurs during the progression of epithelial cancers[13,14]. Recent evidence also indicates that the reduced expression of E-cadherin at cell-cell boundaries correlates significantly with cancer cell dedifferentiation, invasion and metastasis, and thus may be a useful marker of a poor prognosis[15,16].

Several transcription factors have recently been shown to repress E-cadherin. Among these is the zinc-finger protein snail, which is overexpressed in Madin-Darby Canine Kidney Epithelial cells (MDCK) cells exhibiting a motile/invasive phenotype[17,18]. Indeed, overexpression of Snail leads not only to cell detachment and migration but also to reattachment at a second site[19]. Furthermore, Snail contributes to EMT by mediated the downregulation of E-cadherin gene expression via Ras-Raf-MEK-MAPK, PI3K-Akt, TGFβ-Smads, ILK-Akt and Wnt-β-catenin signaling[20-23]. Snail expression has been detected in a large number of human carcinoma and melanoma cell lines[24]. In addition, Snail is expressed at the invasive front of epidermoid carcinomas[25] and is reportedly associated with the invasiveness of ductal breast carcinomas and hepatocarcinomas[26,27]. On the other hand, Snail's stability and subcellular localization are modulated by extracellular factors, including TGFβ and metalloproteinases, suggesting these proteins could potentially serve as targets for the prevention of EMT[28]. Consistent with that idea, Olmeda *et al.* showed that tumors derived from keratinocyte and mammary tumor cell lines in which Snail-1 and/or Snail-2 were knocked down had a lower metastatic potential in nude mice[29].

CRP also has received much attention as a predictive biomarker of cardiovascular disease risk and as a tumor progression factor[30,31]. However, there are as yet no published studies examining the relationship between CRP and E-cadherin or Snail. CRP is produced mainly by hepatocytes in response to acute and chronic inflammation. There are also reports that cancer cells produce CRP locally within the tumor, however they are too low to influence serum CRP levels[2,3]. Prior to tumor migration, primary tumors generally exhibit invasiveness. Our finding demonstrated that CRP significantly slowed NR-S1M cell migration. It means one of the key factors exhibiting invasiveness of primary tumor is present at the host site. Thus inhibition of EMT may be a viable approach to preventing invasion and the early steps of metastasis, and identification of the molecules whose expression is associated with suppression of EMT would be desirable[33,34].

In summary, our findings indicate that CRP suppresses NR-S1M murine squamous cell carcinoma cell migration, perhaps by enhancing expression of E-cadherin. Moreover, these findings suggest that strengthening E-cadherin expression may be a useful approach to preventing cancer invasion and metastasis through suppression of EMT.

Acknowledgements

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