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Molecular Chaperonic Function of C-Reactive Protein Induced by Heating in HT-29 Human Colon Carcinoma Cells

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The effects of heat shock, or *all-trans* retinoic acid, on the expression of the C-reactive protein mRNA in the HT-29 human colon carcinoma cells, as well as the functional role of the C-reactive protein as a molecular chaperone, were studied. The expression level of the C-reactive protein mRNA in the HT-29 cells was increased time-dependently when exposed to heat-shock, and dose-dependently when treated with *all-trans* retinoic acid. The activities of transglutaminase C and K in the HT-29 cells were significantly increased when treated with *all-trans* retinoic acid. The C-reactive protein prevented thermal aggregation of the citrate synthase and stabilized the target enzyme, citrate synthase. The C-reactive protein promoted functional refolding of the urea-denatured citrate synthase up to 40-70%. These results suggest that the C-reactive protein, which is induced in human colon carcinoma cells, when heated or treated with *all-trans* retinoic acid has in a part functional activity of the molecular chaperone.

Keywords: C-reactive protein, Heat shock protein 70A, Heat shock, Molecular chaperone.

Introduction

The C-reactive protein (CRP) is the prototypical serum acute phase protein in most mammalian species. It is synthesized mainly in the liver parenchymal cells (Kushner, 1990; Stadnyk and Gauldie, 1991). CRP is a member of the pentarxin familiy and is composed of five identical noncovalently linked subunits of ~23 Kda. The acute phase response can be elicited from the liver by cytokines, IL-1, IL-6, TNF, IFN, LIF, IL-11 and oncostatin M. During the host response to inflammation or tissue injury, there are many changes in the intermediary metabolism, including a dramatic increase in the concentration of the CRP (Baumann *et al.*, 1990; Baumann *et al.*, 1992; Baumann *et al.*, 1993; Heuertz *et al.*, 1993; Wegenka *et al.*, 1993; Kopf *et al.*, 1994).

There are many functions for CRP that are well recognized. These include: pro-inflammation activity, including complement activation, opsonization and leukocyte activation. It has been reported that cancer cells have self-defense mechanisms for survival against microenvironmental insults by producing multidrug-resistant protein (p-glycoprotein) and heat-shock proteins (Park *et al.*, 2000; Ruth and Roninson, 2000).

In a recent study, Lee *et al.* (1996) identified fibrinogen, one of the acute phase plasma proteins in ME-180 human uterine cervix carcinoma cells. The synthesis of the fibrinogen by the ME-180 cells was measured using [^{35}S] L-methionine incorporation. The expression of mRNA for the Bβ-chain of the fibrinogen in the ME-180 cells, was detected using RT-PCR. It was reported that heat shock element {repetitive sequence $(GT)_{15}G(GT)_3$} that is identical to the bacterial heat shock protein gene exists in the CRP gene promoter (Lei *et al.*, 1985; Woo *et al.*, 1985). Lim (1998) reported that CRP overexpressed HT-29 human colon carcinoma cells by means of transfection of cDNA for CRP, inhibited proliferation, invasion and metastasis *in vitro*.

To date, the functional roles of the CRP in non-hepatic cancer cells has not been established. Therefore the present study was initiated in order to examine the other functions of with the CRP exception of the acute phase reactants.

Particular attention was paid to examine whether or not: (1) The expression of mRNAs for both CRP and heat shock protein-70A (HSP-70A) were upregulated in the HT-29 human colon carcinoma cells when heated. (2) CRP prevented thermal aggregation of citrate synthase (CS) and promoted the functional refolding of the urea-denatured CS.

Materials and Methods

Cell culture and maintenance HT-29 human colon carcinoma

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cells was obtained from American Type Culture Collection (ATCC, Rockville, USA). This cell line was maintained in RPMI-1640 medium (GIBCO, Grand Island, USA) plus 10% fetal calf serum (Hyclone, Logan, USA) and penicillin-streptomycin.

Heating and *all-trans* retinoic acid treatment Exponentially growing cells in culture were centrifuged for 10 min at 800 rpm and resuspended in a medium alone or a medium containing various concentrations of *all-trans* retinoic acid (ATRA, Sigma, St. Louis, USA). Cell suspensions containing 2×10^5 cells/ml of medium alone were heated by placing flasks in a water bath (MC-31, JEIO TECH, Seoul, Korea) at 43.5°C±0.01°C for 30 min.

RNA and DNA amplification procedure (PCR assay) Oligonucleotide primers were synthesized by an automated DNA synthesizer (OPERON). Primer sequences, which were located in exons in a respective gene of human CRP, HSP-70A and β-actin, were as follows; CRP: primer 1 (sense) 5'-TGCCACCAAGAGA CAAGACA-3', primer 2 (antisense) 5'-GCCAGTTCAGGACAT TAGGA-3'; heat shock protein-70A (HSP-70A): primer 1 (sense) 5'-CTAGCCTGAGGAGCTGCTGCGACAG-3', primer 2 (antisense) 5'-GTTCCCTGCTCTCTGTCGGCTCGGCT-3'; β-actin: primer 1 (sense) 5'-GTGGGGCGCCCCAGGCACCA-3', primer 2 (antisense) 5'-CTACCTTAATGTCACGCACGATTTC-3'. cDNA were synthesized from total RNA (6 g/sample) isolated from cancer cells, using random hexamer and avian myeloblastosis virus reverse transcriptase. Following inactivation, A 5 μl sample of cDNA was amplified in a buffer containing 200 nM concentrations each of oligonucleotide primers, 0.1 mM concentrations each of dATP, dCTP, dGTP, dTTP, 1 μCi of [^{32}P] dCTP and 0.1 mM dithiothreitol in a buffer consisting of 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 50 mM KCl, and Taq polymerase (Promega, Madison, USA). The reaction mixture was overlaid with a drop of light mineral oil and PCR was performed in a Perkin Elmer/Cetus DNA thermal cycler for 40-45 cycles. The amplification profile involved denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extention at 72°C for 1 min. A 15 μl sample each of a PCR reaction mixture was electrophoresed in 2% agarose gel containing 0.5 μg/ml of ethidium bromide in 0.5 × TBE buffer. Following electrophoresis, the gel was photographed. Appropriate bands were cut from the gels and radioactivities were counted. The cDNAs derived from the samples were normalized to yield equivalent β-actin products.

Transglutaminase (TGase) assay Cells were plated in 75 cm^2 tissue culture flask and then treated with various concentration of *all-trans* retinoic acid twice 3 days apart. The cells suspended in the reaction mixture contained 1% dimethylated casein, 0.1 M tris-acetate, pH 7.5, 1 mM EDTA, 10 mM $CaCl_2$, 1% Triton X-100 and 4 mM DTT. Following sonication, the supernatant solution (for TGase C) and pellet (for TGase K) were collected by centrifugation. TGase activities were assayed by [1,4-^{14}C] putrescine incorporation into dimethylated casein method. 1 unit of TGases was defined as 1 nM of putrescine into dimethylated casein for 1 h.

Effect of CRP on thermal aggregation of CS The thermal aggregation of the CS was determined by measuring scattering at 360 nm using fluorometer (F-4010, HITACHI, Japan) (Horwitz, 1992; Wiech *et al.*, 1992; Jakob *et al.*, 1993). Light scattering of 150 nM CS dissolved in preheated Buffer A (40 mM HEPES, 20 mM KOH, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM potassium acetate) at 42°C for 10 min in the absence or the presence of 100 nM lysozyme, 30 nM CRP, 50 nM, 25 nM or 12.5 nM α-crystallin, was measured for 40 min.

Dose-dependent effect of CRP on thermal aggregation of CS The thermal aggregation of the CS was determined by measuring absorbance at 360 nm using a spectrophotometer (Hewlett Packard Vetura 830) (Wiech *et al.*, 1992). The absorbance of the 150 nM CS dissolved in the preheated Buffer A at 42°C for 10 min in the absence or presence of 0.2 μM, 0.4 μM or 0.6 μM CRP, or 6 μM lysozyme, was measured for 45 min.

Effect of CRP on the refolding of chemically denatured CS The aggregation of the CS was determined by measuring the scattering at 360 nm using the fluorometer (HITACHI) (Wiech *et al.*, 1992; Jakob *et al.*, 1993). 150 nM of denatured CS by a denaturing buffer (50 mM Tris-HCl, 2 mM EDTA, 8 M urea, 20 mM DTT) at 20°C for 90 min was mixed with 100 nM lysozyme, 50 nM or 100 nM CRP, or 25 nM α-crystallin, followed by 100 fold dilution, and then the scattering was measured.

In these measurements lysozyme was used as a negative control and α-crystallin as a positive control.

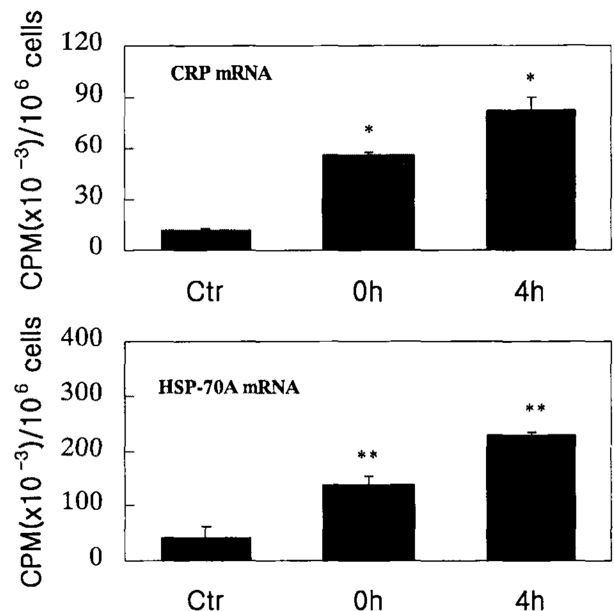

Fig. 1. Expression of CRP and HSP-70A mRNA in HT-29 cells following hyperthermia. cDNAs were amplified using primers for CRP, HSP-70A and β-actin for 45 cycles and PCR products were quantified. Ctr, control cancer cells; 0 h, cancer cells immediately after heating at 43.5°C for 30 min; 4 h, cancer cells incubated for 4 h after heating at 43.5°C for 30 min. *P<0.0001; **P<0.001.

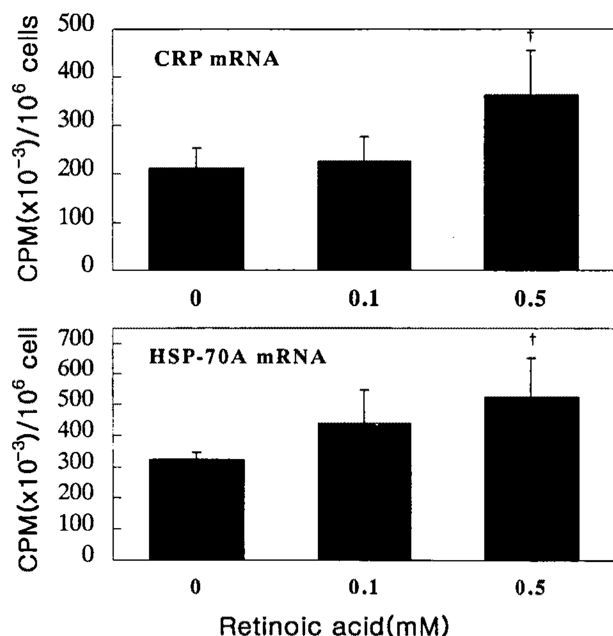

Fig. 2. Expression of CRP and HSP-70A mRNA in HT-29 cells treated with *all-trans* retinoic acid. cDNAs were amplified using primers for CRP, HSP-70A and β-actin for 48 cycles. CRP and HSP-70A PCR products were quantified. †P<0.05.

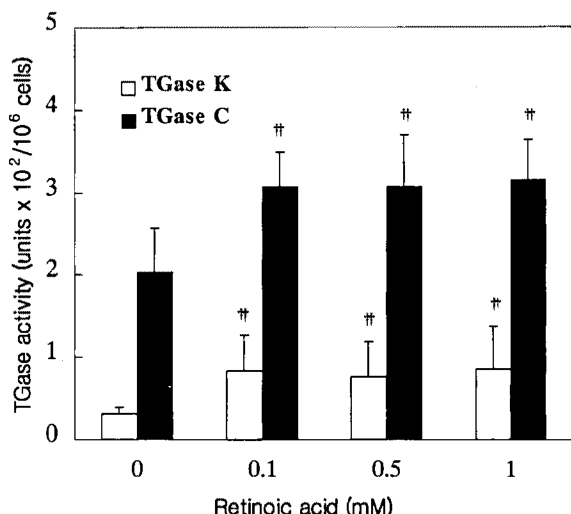

Fig. 3. Transglutaminases activities in HT-29 cells treated with *all-trans* retinoic acid. ††P<0.005.

Results

As shown in Fig. 1, mRNA levels of CRP, as well as those of HSP-70A in HT-29 cells when heated, were upregulated time-dependently. This indicates that the regulation of expression of the CRP mRNA in HT-29 cells was similar to that of HSP-70A when heated.

These results allowed studies on the effects of ATRA, which has been known to be one of differentiating agents, on the expression of CRP and HSP-70A mRNA in HT-29 cells to be initiated. As shown in Fig. 2, the mRNA levels, as well as

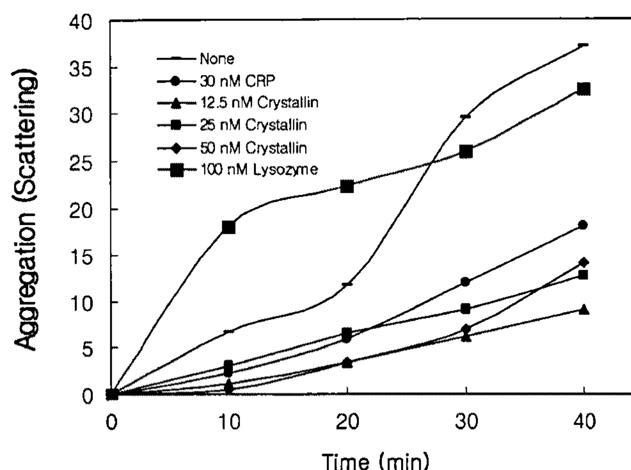

Fig. 4. Effect of CRP on thermal aggregation of CS. The kinetics of aggregation were determined by light scattering. 150 nM CS was equilibrated at 42°C, in the absence or the presence of 100 nM lysozyme, 30 nM CRP, 50 nM, 25 nM or 12.5 nM α-crystallin.

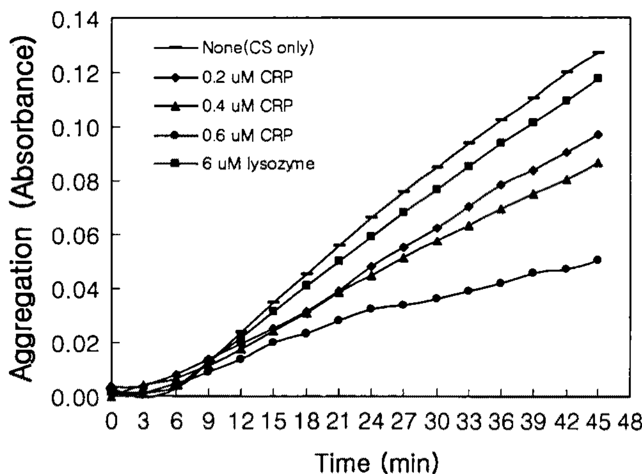

Fig. 5. Dose-dependent effect of CRP on thermal aggregation of CS. The kinetics of aggregation were determined by absorbance. 150 nM CS was equilibrated at 42 °C, in the absence or the presence of 0.2 μM, 0.4 μM, 0.6 μM CRP or 6 μM lysozyme.

those of HSP-70A in HT-29 cells when treated with ATRA, were upregulated dose-dependently.

As shown in Fig. 3, TGase K and TGase C activities, which have been well recognized as being increased in terminally well-differentiated cells, were also increased when treated with ATRA.

HSP-70A has been understood to be one of the molecular chaperones. In order to examine the molecular chaperonic activity of the CRP, the inhibitory effects of the CRP on thermal aggregation of the CS, target enzyme, and the effect of the CRP on the refolding of chemically denatured CS, were studied.

As shown in Fig. 4, the CRP inhibited thermal aggregation

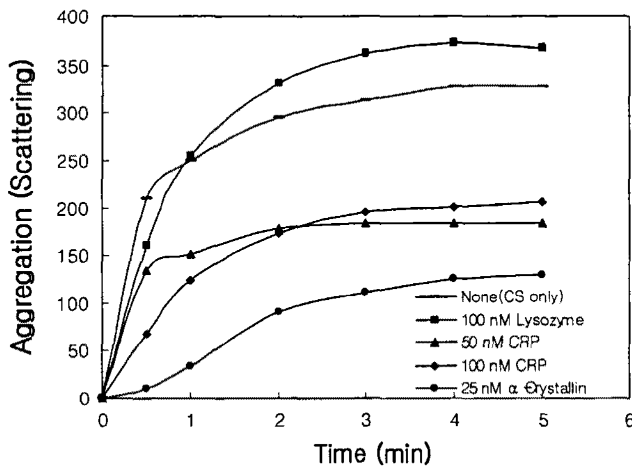

Fig. 6. Effect of CRP on the refolding of chemically denatured CS. Kinetics of aggregation of CS in the absence or the presence of 100 nM lysozyme, 50 nM or 100 nM CRP, or 25 nM α-crystallin were measured.

of the CS. The inhibitory effect of the CRP on thermal aggregation of the CS was similar to that of α-crystallin which has long been recognized as a model of molecular chaperone.

As shown in Fig. 5, the inhibitory effect of the CRP on the thermal aggregation of the CS was dose-dependent.

As shown in Fig. 6, the CRP refolded the chemically denatured CS up to 40-60% of that of the α-crystallin, the positive control of the molecular chaperone.

Discussion

In the present study, the time-dependently upregulated expression of the CRP mRNA in HT-29 cells when heated at 43°C for 30 min, was similar to that of HSP-70A. This result was provided by evidence that the 5' portion of the CRP gene has 3 regions similar to Drosophila heat shock consensus sequence (Woo *et al.*, 1985). It is recognized that the HSP-70A is one of the molecular chaperones and the HSP-70 family consists of Dnak, Ssal-4p, Hsc73, Kar2p, BiP/Grp78, Ssclp, ctHSP70 (Chirico *et al.*, 1988; Amir-Shapira *et al.*, 1990; Hendrick and Hartl, 1993).

In this study, both the mRNAs for CRP and HSP-70A in the HT-29 cells were dose-dependently upregulated when treated with ATRA. It has been established that TGase K and TGase C activities were elevated in terminally differentiated cells (Scott *et al.*, 1982), and that the HT-29 cells were significantly increased when treated with ATRA. It is recognized that ATRA is one of the cell differentiating agent and that TGase K and TGase C activities are increased in the differentiated cells. These results are supported by the evidence that the differentiated HT-29 cells, when cultured on polycarbonate membrane, upregulatedly expressed the CRP mRNA (Choi, 1997). Also, the CRP-overexpressed HT-29 cells that were transfected with CRP-cDNA weakened the malignant characteristics of the cancer cells, it's proliferation, invasion and metastasis (Lim, 1998).

These evidences allowed studies on the molecular chaperonic function of the CRP to be initiated. In the present study we learned: (1) The CRP had an inhibitory effect on the thermal aggregation of the CS, which has been well understood to be a target enzyme for the examination of the molecular chaperone. (2) The inhibitory activity of the CRP on the thermal aggregation of the CS is dose-dependent. (3) The CRP had up to 40-60% of the refolding activity of the chemically denatured CS when compared with that of the α-crystallin which has been known to be a model of the molecular chaperones. (4) As reported by Lee *et al.* (2000), the validity of the RT-PCR confirmed that the mRNA levels examined by the RT-PCR coincided with those of synthesized proteins.

In conclusion, the CRP that was induced in HT-29 human colon carcinoma cells by heat shock has, in part, a functional activity of the molecular chaperone.

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