

# Cytokine expression in human dermal fibroblasts stimulated with eosinophil cationic protein measured by protein array

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## Summary

**Background:** Eosinophil cationic protein (ECP) was reported previously to be involved in allergic inflammation with cytotoxic activity. On the other hand, recent studies showed that ECP did not induce cell death but inhibited the growth of cancer-derived cells. Our previous study indicated that human ECP enhanced differentiation of rat neonatal cardiomyocytes and stress fiber formation in Balb/c 3T3 mouse fibroblasts, while the effects of human ECP on human fibroblasts are unknown.

**Objective:** The present study was performed to determine the effects of human ECP on cytokine expression in human fibroblasts by protein array.

**Methods:** The effects of recombinant human ECP (rhECP) on normal human dermal fibroblasts (NHDF) were examined by assaying cell growth. Furthermore, cytokine expression of NHDF stimulated by ECP, which could influence cell growth, was evaluated by protein array.

**Results:** ECP was not cytotoxic but enhanced the growth of NHDF. The peak rhECP concentration that enhanced the cell counts by 1.56-fold was 100 ng/mL, which was significantly different from cultures without ECP stimulation (ANOVA/Scheffe's test,  $P < 0.05$ ). Array analyses indicated that ciliary neurotrophic factor (CNTF), neutrophil-activating peptide (NAP)-2, and neurotrophin (NT)-3 were significantly upregulated in NHDF stimulated with 100 ng/mL ECP compared to those without stimulation.

**Conclusion:** ECP is not cytotoxic but enhances the growth of NHDF. CNTF, NAP-2, and NT-3 were suggested to be involved in enhancing the growth of NHDF. These findings will contribute to determination of the role of ECP in allergic inflammation. (*Asian Pac J Allergy Immunol* 2013;31:271-6)

**Key words:** cytokine, eosinophil cationic protein, fibroblast, growth

## Introduction

Eosinophil cationic protein (ECP) is one of the proteins released on eosinophil degranulation, and is generally recognized as being involved in allergic inflammation with cytotoxic activity. This cytotoxicity is mainly explained as an antiparasitic and bactericidal activity in infectious diseases. However, ECP has also been reported to be cytotoxic toward host cells, e.g., the tracheal epithelium.<sup>1</sup>

On the other hand, recent studies showed that ECP has a broad impact on cell functions. ECP was reported to show neurotoxicity<sup>2</sup> and apoptotic effects on several eukaryotic cell lines,<sup>3</sup> while it did not induce cell death but inhibited the growth of cancer-derived cells.<sup>4</sup> Furthermore, human ECP enhanced differentiation of rat neonatal cardiomyocytes and stress fiber formation in Balb/c 3T3 mouse fibroblasts,<sup>5</sup> while the effects of human

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Submitted date: 14/5/2012

Accepted date: 25/12/2012

ECP on human fibroblasts are unknown. Despite their usefulness in determination of eosinophil physiology *in vivo*, murine models differ from humans in several major respects.<sup>6</sup> For example, airway mucosal eosinophils in allergen-challenged mice lack signs of degranulation<sup>7</sup>. Thus, the role of human ECP could be different from that in rodents. Therefore, it is important to evaluate the effects of human ECP on human fibroblasts. ECP is detected in allergic inflammatory lesions, and is widely used as a biomarker for the activity of allergic inflammation. Thus, ECP is generally considered to be involved in allergic pathology. Evaluation of cytokine expression in human fibroblasts stimulated by ECP could reveal the role of ECP in allergic lesions. However, little information is available regarding cytokine expression of human fibroblasts stimulated by ECP. One study showed that ECP stimulates transforming growth factor (TGF)- $\beta$ 1 release by human lung fibroblasts *in vitro*,<sup>8</sup> although the cytokine production profile from fibroblasts stimulated by ECP has not been determined in detail.

In the present study, we examined the effects of recombinant human ECP (rhECP) on normal human dermal fibroblasts (NHDF) by assay for cell growth. Furthermore, cytokine expression of NHDF stimulated by ECP, which could influence cell growth, was evaluated by protein array.

## Methods

### Recombinant human ECP

Recombinant human ECP (rhECP) in mature form without the secretion signal peptide was expressed in bacteria and prepared as described previously.<sup>9</sup> Briefly, human ECP cDNA was isolated and expressed in an *Escherichia coli* T7 expression system. Purified rhECP was assessed for RNase activity on yeast RNA by the perchloric acid precipitation method<sup>10</sup> and for bactericidal activity.<sup>11</sup> The amino acid sequence, CD spectra, and apparent molecular mass on SDS-PAGE were confirmed. No endotoxin was detected by limulus test. rhECP was of sufficiently high purity that crystals could be obtained, and these were used to determine the 3D structure at high resolution.<sup>9</sup> Therefore, this preparation should not include any contaminants derived from bacterial cells.

### Culture of NHDF

Commercially available adult NHDF (Lonza Walkersville, Inc., Walkersville, MD) were maintained

in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10  $\mu$ g/mL gentamycin at 37°C under a humidified 5% CO<sub>2</sub> atmosphere. All *in vitro* studies using NHDF were performed between passages 5 and 9.

### Microscopic observation and cell counts of NHDF stimulated with rhECP

NHDF were plated into 8-well chamber slides (Lab-Tek™ Chambered Coverglass; Nalge-Nunc, Rochester, NY) with DMEM containing 10% FBS and 10  $\mu$ g/mL gentamycin at  $5.0 \times 10^3$  cells/well. After 24 h, the medium was changed to DMEM containing 0.5% FBS without gentamycin. After a further 24 h, cells were stimulated with rhECP (0–10  $\mu$ g/mL) for 24 h. The cells were stained with hematoxylin-eosin, and microscopic observation of NHDF with or without rhECP (0–10  $\mu$ g/mL) stimulation was performed. Furthermore, the number of NHDF cells was counted at a magnification of  $\times 100$ . Five separate fields from a single well were counted, and five separate wells were used for each rhECP concentration group. Averages and standard deviations were calculated from three independent experiments.

### Cytokine measurements

NHDF were plated into 6-well multiple well plates (Corning, Lowell, MA) with DMEM containing 10% FBS and 10  $\mu$ g/mL gentamycin at  $1.0 \times 10^5$  cells/well. After 24 h, the medium was changed to DMEM containing 0.5% FBS without gentamycin. After a further 24 h, cells were stimulated with or without 100 ng/mL rhECP for 24 h.

Culture supernatants were obtained, and cytokine measurements were performed by Human Cytokine Antibody Array C series 1000 (Ray Biotech Inc, Norcross, GA), consisting of 120 different cytokine and chemokine antibodies spotted in duplicate onto a membrane according to the manufacturer's instructions. The array was exposed to film (Amersham Biosciences, Buckinghamshire, UK) and the intensity of signals was detected using ImageJ software (NIH, Bethesda, MD). The relative intensity levels of the cytokines were normalized with reference to the amounts present on the positive control in each membrane based on the average of cytokine spot intensity levels divided by the average of the positive control spot intensity levels and are shown as percentages. The sensitivities of the cytokines examined are provided on the manufacturer's

website (<http://www.raybiotech.com/Resources.asp?m=6>). Average and standard deviations were calculated from three independent experiments.

### Statistical analysis

All statistical analyses were performed using SPSS statistical software version 13.0 (SPSS, Chicago, IL). The cell counts for NHDF stimulated with each concentration of ECP were compared to those without ECP stimulation using ANOVA/Scheffe's test. The results of Cytokine Antibody Array with and without ECP stimulation were compared using Welch's *t* test. In all analyses,  $P < 0.05$  was taken to indicate statistical significance.

## Results

### Microscopic observation and cell counts of NHDF stimulated with rhECP

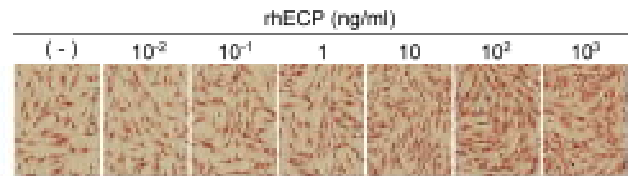
The results of microscopic observation are shown in Figure 1. ECP was not cytotoxic toward NHDF, but rather enhanced the growth of these cells. Cell counts of NHDF stimulated with rhECP are shown in Figure 2. A dose-dependent increase in cell count was observed. The peak rhECP concentration that enhanced the cell counts by 1.56-fold was 100 ng/mL, which was significantly different from cultures without ECP stimulation (ANOVA/Scheffe's test,  $P < 0.05$ ).

### Cytokine expression in NHDF stimulated with rhECP

The relative cytokine levels released from NHDF with or without rhECP stimulation are summarized in Table 1. The array analyses indicated that ciliary neurotrophic factor (CNTF), neutrophil-activating peptide (NAP)-2, and neurotrophin (NT)-3 were significantly upregulated in NHDF stimulated with rhECP compared to those without stimulation (Welch's *t* test,  $P < 0.05$ ).

## Discussion

The results of the present study indicated that ECP is not cytotoxic but enhances the growth of NHDF, which was consistent with our previous observations in Balb/c 3T3 mouse fibroblasts. The peak rhECP concentration that enhanced the cell count was 100 ng/mL, and therefore we performed subsequent cytokine expression analysis of NHDF by protein array at this rhECP concentration. The results of array analyses indicated that CNTF, NAP-2, and NT-3 were significantly upregulated in

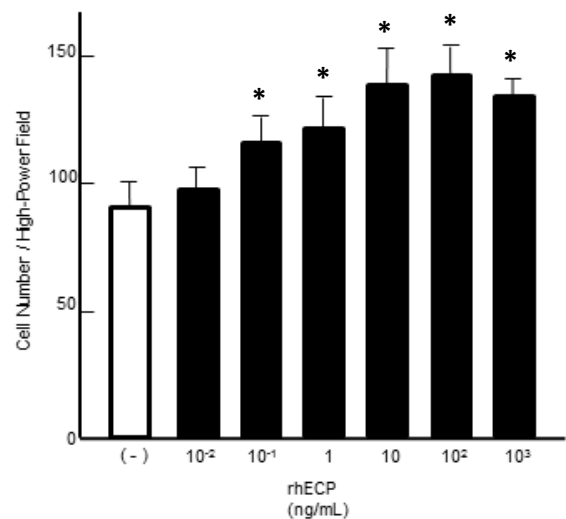


**Figure 1.** Microscopic observation ( $\times 100$ ) of NHDF with or without rhECP ( $10^{-2}$  –  $10^3$  ng/mL) stimulation for 24 h. NHDF were stained with hematoxylin-eosin.

NHDF with ECP stimulation compared to unstimulated controls.

CNTF is a member of IL-6 cytokine family, which promotes the maintenance, differentiation, and/or survival of a variety of neurons within the nervous system.<sup>12</sup> In addition to the effects on the nervous system, CNTF also acts on the immune system. Interestingly, it has been reported that CNTF enhances IgE production by B cells from atopic patients.<sup>13</sup> CNTF derived from NHDF by ECP stimulation may contribute to the elevation of IgE level in allergic inflammation.

NAP-2 is the chemotactically most active form of chemokine (C-X-C motif) ligand (CXCL)7. CXCL7 is the most abundant of the chemokines stored and secreted by platelets,<sup>14</sup> while our results



**Figure 2.** Cell counts of NHDF with or without rhECP ( $10^{-2}$  –  $10^3$  ng/mL) stimulation for 24 h. rhECP concentrations in the range  $10^{-1}$  –  $10^3$  ng/mL significantly enhanced the number of NHDF cells compared to unstimulated controls ( $*P < 0.05$ , ANOVA/Fisher's PLSD). The peak rhECP concentration that enhanced the cell count by 1.56-fold was 100 ng/mL.

**Table 1.** Relative cytokine levels

Cytokine	Relative cytokine levels to positive control (%)		Fold increase
	ECP (-)	ECP (+)	
Angiogenin	10.0±4.6	13.3±9.4	1.33
BDNF	12.0±7.4	12.1±3.3	1.01
BLC	4.4±1.8	5.5±2.6	1.26
BMP-4	9.8±6.0	10.2±4.2	1.04
BMP-6	6.9±1.8	8.3±1.0	1.2
CKb8-1	11.4±4.4	14.6±2.4	1.28
CNTF	7.4±0.5	8.9±0.5*	1.21
EGF	19.0±5.4	19.6±1.4	1.03
Eotaxin	11.7±10.1	7.6±1.6	0.65
Eotaxin-2	11.5±2.2	8.8±4.8	0.77
Eotaxin-3	6.6±2.5	5.4±3.0	0.82
FGF-6	11.0±4.9	10.3±4.4	0.94
FGF-7	7.9±6.7	8.5±5.6	1.06
Fit-3 Ligand	4.6±2.9	6.4±3.9	1.4
Fractalkine	5.7±3.3	6.5±2.5	1.15
GCP-2	5.7±2.8	5.6±2.3	0.98
GDNF	8.8±3.0	9.2±3.2	1.04
GM-CSF	2.2±0.9	2.1±1.3	0.95
I-309	6.8±1.3	7.1±1.7	1.05
IFN-g	6.1±0.1	7.6±1.2	1.25
IGFBP-1	4.8±2.1	6.9±3.8	1.43
IGFBP-2	16.6±4.6	16.6±3.9	1
IGFBP-4	19.4±25.9	17.2±20.3	0.89
IGF-1	4.8±3.2	3.5±1.6	0.73
IL-10	5.4±0.4	3.5±1.0	0.64
IL-13	1.7±1.0	1.5±1.6	0.89
IL-15	7.4±5.1	7.6±4.8	1.02
IL-16	5.1±3.4	6.0±3.0	1.17
IL-1 $\alpha$	5.7±2.2	7.6±4.8	1.34
IL-1 $\beta$	5.9±1.4	5.9±1.9	1
IL-1ra	14.6±2.6	12.8±3.2	0.88
IL-2	4.3±2.3	4.0±1.5	0.94
IL-3	5.6±1.8	5.9±1.8	1.05
IL-4	4.3±1.5	5.0±1.8	1.14
IL-5	4.6±5.0	8.2±9.8	1.77
IL-6	41.2±65.9	46.9±73.2	1.14
IL-7	5.6±8.1	3.3±3.3	0.59
LEPTIN(OB)	9.6±3.6	7.4±3.7	0.77
LIGHT	8.0±2.7	9.3±2.8	1.16
MCP-1	22.4±20.9	25.6±29.1	1.14
MCP-2	5.9±4.0	6.4±3.5	1.08
MCP-3	1.9±0.3	2.4±1.8	1.28
MCP-4	4.3±1.0	4.6±2.2	1.06
M-CSF	11.9±2.4	11.5±0.5	0.96
MDC	12.2±3.7	8.8±1.4	0.72
MIG	8.0±3.9	8.1±2.1	1.01
MIP-1d	4.4±3.7	4.9±2.5	1.12
MIP-3a	6.3±3.1	6.6±1.7	1.04
NAP-2	15.0±1.0	18.9±1.4*	1.25
NT-3	15.7±2.3	21.0±2.8*	1.34
PARC	13.4±12.8	13.3±8.0	0.99
PDGF-BB	7.7±2.8	5.9±4.4	0.76
RANTES	11.6±5.2	15.1±3.8	1.3
SCF	8.2±5.4	11.1±3.6	1.35
SDF-1	16.1±12.1	18.6±6.0	1.16
TARC	10.9±6.9	11.0±4.5	1.01
TGF-b1	7.2±2.8	7.0±3.5	0.96
TGF-b3	10.3±1.4	9.6±1.7	0.93
TNF-a	9.8±7.0	7.9±4.4	0.81
TNF-b	13.9±2.8	15.7±5.2	1.13

**Table 1.** Continued

Cytokine	Relative cytokine levels to positive control (%)		Fold increase
	ECP (-)	ECP (+)	
Adiponectin/Acp30	5.9±3.6	5.8±4.8	0.98
AgRP (ART)	12.4±6.5	8.9±7.6	0.72
Angiopoietin-2	14.7±6.9	10.1±9.1	0.69
AR (Amphiregulin)	11.3±5.2	7.7±6.6	0.68
Axl	11.8±3.3	9.4±4.6	0.79
bFGF	10.0±5.6	6.9±5.1	0.69
NGF-b	8.2±4.0	5.8±3.7	0.71
BTC	11.6±2.2	10.0±4.4	0.87
CCL28/VIC	5.9±3.1	4.5±2.3	0.76
CTACK/CCL27	6.5±5.4	9.9±3.3	1.52
Dtk	9.0±8.9	9.8±5.7	1.09
EGF-R	4.3±3.7	4.4±3.6	1.04
ENA-78	6.9±4.3	7.7±5.2	1.11
Fas/TNFRSF6	17.1±9.0	17.3±8.6	1.01
FGF-4	25.6±6.3	26.9±10.5	1.05
FGF-9	17.6±10.2	16.4±11.3	0.93
GCSF	6.6±1.4	4.4±0.8	0.67
GITR Ligand/TNFSF18	9.2±3.6	7.0±4.3	0.76
GITR/TNFRF18	12.7±4.4	10.1±5.4	0.8
GRO	34.0±5.8	30.9±5.5	0.91
GRO-a	7.3±2.8	6.0±3.8	0.82
HCC-4/CCL16	10.9±4.3	9.1±5.0	0.83
HGF	9.6±5.6	13.4±12.2	1.39
ICAM-1	7.4±5.7	10.3±5.7	1.38
ICAM-3	5.9±4.4	6.3±4.1	1.07
IGFBP-3	13.7±8.5	12.2±7.2	0.89
IGFBP-6	24.3±4.7	24.0±6.9	0.99
IGF-1sR	10.8±6.6	10.5±8.5	0.97
IL-1R4/ST2	12.8±6.8	12.9±7.0	1.01
IL-1sRI	8.7±5.1	7.9±5.1	0.91
IL-11	4.2±2.3	2.9±1.4	0.7
IL-12p40	20.4±8.0	18.5±9.0	0.91
IL-12p70	11.8±4.4	9.2±6.8	0.78
IL-17	5.3±2.2	3.7±2.4	0.69
IL-2Ralpha	10.0±3.3	9.0±6.3	0.9
IL-6sR	14.7±1.6	14.0±4.8	0.95
IL-8	12.8±2.5	13.0±4.1	1.01
I-TAC/CXCL11	6.7±6.0	9.5±5.7	1.41
Lymphotactin	10.0±5.2	10.6±5.8	1.06
MIF	18.4±6.0	18.9±8.4	1.02
MIP-1a	13.2±6.9	13.2±8.3	1
MIP-1b	11.0±7.0	10.7±7.2	0.97
MIP-3b	4.1±2.7	3.5±2.5	0.85
MSP a	19.0±9.5	18.9±8.9	1
NT-4	10.4±3.3	9.5±4.1	0.91
Osteoprotegerin	43.8±11.1	41.6±11.7	0.95
ONCOSTATIN M	11.6±5.8	10.6±7.9	0.91
PIGF	7.3±2.3	6.5±4.0	0.89
sgp130	12.9±3.1	11.7±5.2	0.91
sTNFRII/TNFRS1B	13.9±3.0	12.2±6.5	0.88
sTNFRI/TNFRS1A	19.0±6.4	16.0±6.6	0.84
TECK/CCL25	5.9±5.1	8.1±4.8	1.37
TIMP-1	72.1±2.5	63.3±10.8	0.88
TIMP-2	69.5±12.0	62.0±14.2	0.89
TPO	4.7±5.3	4.4±4.0	0.94
TRAILsR3/TNFRS10C	8.2±5.0	7.8±5.7	0.95
TRAILR4/TNFRS10D	7.3±3.9	7.0±4.8	0.97
uPAR	17.6±6.4	16.0±6.0	0.91
VEGF	9.9±4.8	10.0±5.8	1.01
VEGF-D	12.7±7.1	8.9±11.4	0.7

indicated that ECP stimulated NHDF to secrete one of the variants of CXCL7, i.e., NAP2. There are several known molecular variants of CXCL7, including platelet basic protein (PBP), connective tissue-activating peptide III (CTAP-III),  $\beta$ -thromboglobulin ( $\beta$ -TG), and NAP-2.<sup>14</sup> These CXCL7 variants are proteolytically derived from a precursor molecule (pre-platelet basic protein; pre-PBP) encoded by the CXCL7 gene.<sup>14</sup> It has been reported that both CTAP-III and NAP-2, as well as other truncated CXCL7 variants of intermediate size, all have the capacity to support various aspects of fibroblast metabolism, e.g., synthesis of matrix components, such as hyaluronic acid and glycosaminoglycans (GAGs),<sup>15</sup> enhancement of GLUT-1 glucose transporter expression, and concomitant glucose uptake.<sup>16</sup> Based on these previous reports, our results indicate a mechanism by which ECP stimulates NHDF to enhance their growth; i.e., autocrine activation of ECP stimulated NHDF via NAP-2 produced by themselves.

NT-3 is a member of the neurotrophin (NT) family, which are growth factors originally described as critical trophic factors involved in the regulation of neuronal development, survival, and function, including synapse formation and plasticity.<sup>17</sup> On the other hand, elevated serum levels of NTs have been found in patients with various allergic diseases, including asthma, allergic rhinitis, and atopic dermatitis.<sup>17</sup> It was reported that NTs, including NT-3, were produced endobronchially following allergen provocation, and they were suggested to contribute to the pathogenesis of asthma.<sup>18</sup> It is not surprising that ECP induces NT-3 production by fibroblasts.

All three significantly upregulated molecules—CNTF, NAP-2, and NT-3—could explain the involvement of ECP in allergic inflammation and the promotion of growth of NHDF. Indeed, while not particularly high (1.56-fold), the enhancement of cell count by ECP stimulation was observed. These findings regarding ECP activity suggest that ECP functions in fibrosis with mild promotion of allergic inflammation. Other molecules that were upregulated below the level of significance on array analysis may also contribute to this phenomenon in a cooperative manner. Our results also indicated that the levels of production of major proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , etc., were not significantly increased by ECP stimulation. Taken together, these observations indicated that

ECP is not cytotoxic and does cause a strong inflammatory reaction in NHDF.

Zagai et al. reported that ECP stimulates TGF- $\beta$ 1 release by human lung fibroblasts.<sup>8</sup> However, their experiments were performed with ECP stimulation at doses of 1–10  $\mu$ g/mL. Significant elevation of TGF- $\beta$ 1 release was observed with ECP stimulation at concentrations > 5  $\mu$ g/mL, and was not observed at 1  $\mu$ g/mL. Our experiment was performed with ECP stimulation at a dose below 1  $\mu$ g/mL (i.e., 100 ng/mL). Our observation that there was no significant change in TGF- $\beta$ 1 release from NHDF by ECP stimulation is not in conflict with the report of Zagai et al.<sup>8</sup>

In conclusion, ECP is not cytotoxic but enhances the growth of NHDF. The results of array analyses indicated that CNTF, NAP-2, and NT-3 were significantly upregulated in NHDF with ECP stimulation compared with unstimulated controls. These findings could contribute to determination of the role of ECP in allergic inflammation.

#### Acknowledgements

This study was supported by a Grant-in-Aid for Young Scientists (B #22791836 and # 24792024 to YS) from the Japan Society for the Promotion of Science.

#### Conflict of interest

We have no conflicts of interest related to this study.

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