Table II. Response to gefitinib therapy.

| | CR | PR | SD | PD | NE |
|---|---|---|---|---|---|
| No. of patients | 0 | 8 | 6 | 5 | 5 |
| Median TTP (months) | | 16.1 | 9.3 | 1.0 | |
| Median OS (months) | | 25.9 | 20.8 | 6.5 | |

TTP, time to progression; OS, overall survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated.

showed weak membrane staining, 2 (weakly positive) when >10% showed complete membrane staining although with weak to moderate intensity and 3 (strongly positive) when >10% had complete and strong membrane staining. Entire tumors with staining scores of 0 or 1 were considered negative while those scored as 2 or 3 were considered positive.

*Statistical analyses.* All statistical analyses were performed by StatView version 5 software (SAS institute Inc, Cary, NC). Comparisons of the proportions between two populations utilized the $\chi^2$ test. Comparisons of patient outcome (TTP and overall survival, OS) between patient groups utilized the Kaplan-Meier method and the log-rank test. All statistical tests were two-sided and $P<0.05$ was considered significant.

## Results

*Response to the gefitinib and patient outcome.* The responses to gefitinib are summarized in Table II. The responders [complete response: CR + partial response: PR, (8/24) 33%] had significantly longer TTP and OS than non-responders ($p<0.005$ and $p<0.05$, respectively). In addition, the patients with controlled disease [CR + PR + stable disease: SD (13/24) 54%] had significantly longer TTP and OS ($p<0.001$ and $p<0.001$, respectively). We found no significant differences in the OS between patients with PR and SD nor could we prove that CR + PR better defined patients who benefited from the therapy than CR + PR + SD. We therefore performed the analyses based on the two groupings.

*Analyses of the EGFR mutation status and staining of p-EGFR, p-Akt and E-cadherin.* We investigated the EGFR mutation status and the staining of p-EGFR, p-Akt and E-cadherin. We chose these proteins because they are intimately connected with the activity of EGFR and thus may predict responsiveness to gefitinib and/or patient outcome. In the mutation analysis, 10 patients were found to have an EGFR mutation: one had a point mutation L858R(T2573G), two had a deletion E746-A750del(2235-2249del), six had a deletion E746-A750del (2236-2250del), and one had a deletion L747-S752del, P753S(2240-2257del). All these mutations have been observed in gefitinib responders in the literature (2,3). Representative immunohistochemical staining is shown in Fig. 1 with the results summarized in Table III. The results of the EGFR mutation status are also shown. Positive p-Akt staining was associated with EGFR mutation, which is plausible because mutant EGFR stimulates the cell survival signal that is mediated by p-Akt. The staining intensity of p-EGFR and E-cadherin failed to show an association with the EGFR mutation and thus may be an independent parameter.

*Predictors of the responsiveness to gefitinib.* We then investigated the association between the expression of these proteins and the responsiveness to gefitinib (Table IV). The presence of an EGFR mutation significantly associates with responsive diseases (CR + PR) or controlled diseases (CR + PR + SD). This is consistent with the results presented in previous reports (20-22). We found no significant associations in the staining result for p-EGFR, p-Akt and E-cadherin.

*Predictors of patient outcome.* We compared the Kaplan-Meier curves to identify predictors of longer TTP and/or OS. As shown in Fig. 2A the positive staining of E-cadherin predicts a longer TTP (12.4 vs. 5.9 months, $p<0.05$) and longer OS (18.4 vs. 13.0 months, $p<0.05$). The presence of EGFR mutation(s) ($p=0.13$ and $p=0.11$, respectively, Fig. 2B), as well as p-EGFR and p-Akt staining intensity failed to predict outcome. We then looked at the EGFR mutation status in conjunction with the E-cadherin staining intensity as predictors of these same parameters. As shown in Fig. 2C in the right panel, the patients with EGFR mutation-positive tumors and those with E-cadherin-positive tumors defined a patient group with a median OS of 18.4 months and excluded the patient group with the median OS of 3.7 months, although we failed to show a significant difference in TTP (Fig. 2C, left panel). Therefore, we consider that the patients with EGFR mutation-positive or E-cadherin-positive tumors are the most likely to benefit from gefitinib therapy.

## Discussion

It was shown that NSCLC tumors with an EGFR mutation(s) respond to gefitinib at a rate of 65 to 100% (5-7,20-24). Several prospective phase II studies have shown that gefitinib therapy significantly lengthened TTP in NSCLC patients with EGFR mutation-positive tumors (5-7). Thus far, no prospective studies have reported on OS. Several retrospective studies have suggested that gefitinib therapy may result in a longer OS in patients with EGFR mutation-positive tumors (20,21,23), however, we did not observe any significant differences in either TTP or OS. This is likely due to the size of the current study, as is discussed later.

We showed that positive E-cadherin staining is significantly associated with TTP and OS. Possible mechanisms that may explain this observation include that i) tumors with a lower E-cadherin expression progress faster than those with a higher expression and ii) E-cadherin modifies EGFR function and thus contributes to the effect of gefitinib treatment. The former mechanism is supported in reports that show that tumors with a positive E-cadherin staining are more frequent in early stage than in locally advanced or metastasizing NSCLCs (25-28). Similar results have been obtained in other malignancies such as the esophagus (29,30), stomach (31,32), colon (33),

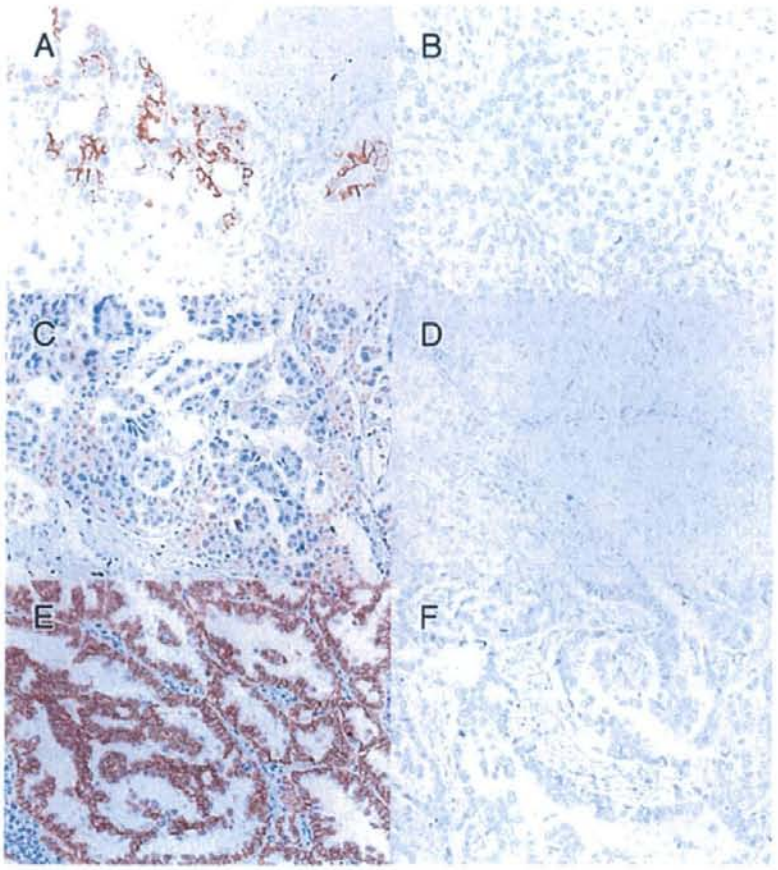


Figure 1. Immunohistochemistry. Positive (A) and negative (B) staining for p-EGFR; positive (C) and negative (D) staining for p-Akt; positive (E) and negative (F) staining for E-cadherin; magnification, x200.

Table III. EGFR mutation and staining of p-EGFR, p-Akt and E-cadherin.

| | p-EGFR | | p-Akt | | E-cadherin | |
|---|---|---|---|---|---|---|
| | Positive | Negative | Positive | Negative | Positive | Negative |
| All patients | 3 | 21 | 3 | 21 | 19 | 5 |
| EGFR mutation | | | | | | |
| Positive | 2 | 8 | 3 | 7 | 9 | 1 |
| Negative | 1 | 13 | 0 | 14 | 10 | 4 |
| P | 0.35 | | <0.05 | | 0.27 | |

EGFR, epidermal growth factor receptor; p-EGFR, phosphorylated-EGFR; p-Akt, phosphorylated-Akt.

liver (34), pancreas (35) and urinary bladder (36,37). Moreover, in NSCLCs, a positive E-cadherin expression associates with a more differentiated histology (26,28) and a better prognosis (25,27,28). The latter mechanism is supported by reports showing that E-cadherin interacts with EGFR, thereby decreasing ligand-affinity (38,39) and inhibiting activation (40) in several human tumor types including the esophageal, breast and lung (41-43). Mechanisms i) and ii) stated above are not mutually exclusive and both may contribute to a better prognosis.

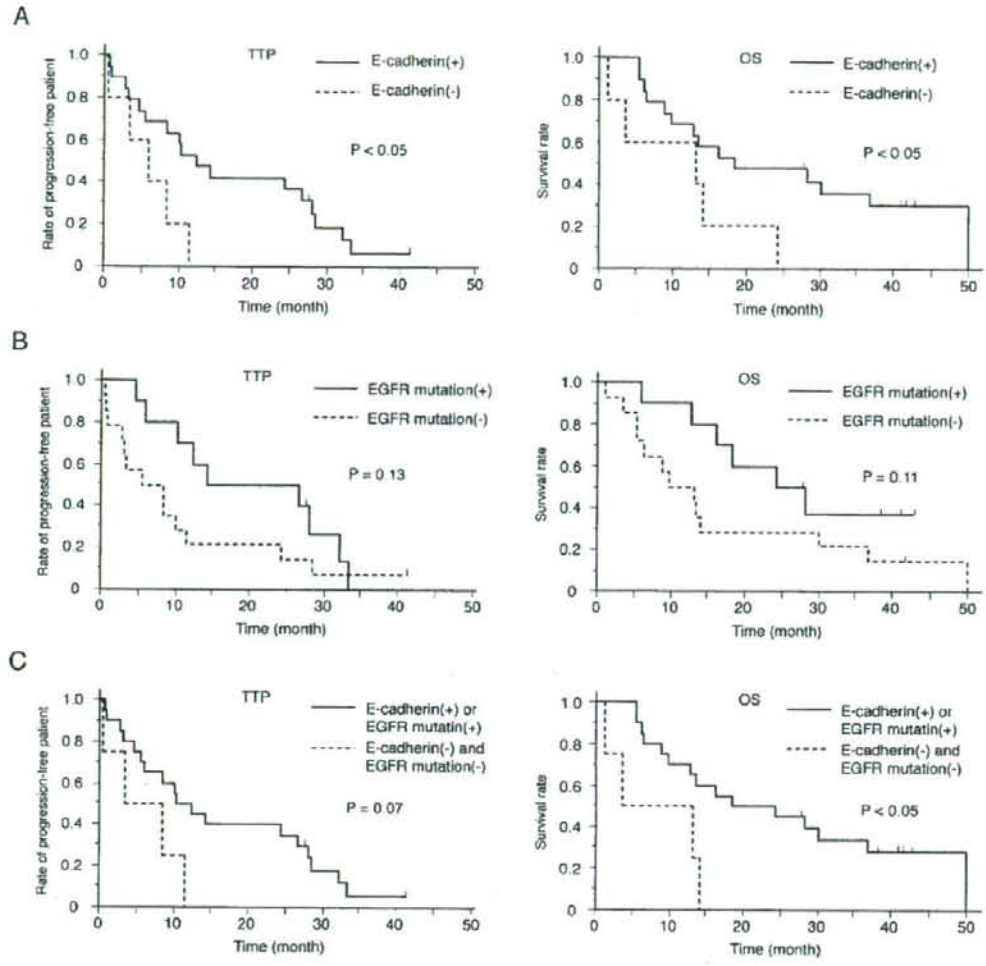


Figure 2. (A) Kaplan-Meier plots of TTP and OS where patients are grouped by the E-cadherin staining of their tumors. (B) Kaplan-Meier plots of TTP and OS where patients are grouped by the EGFR mutation status of their tumors. (C) Kaplan-Meier plots of TTP and OS where the two groups of patients have i) tumors which stain positively for E-cadherin or have an EGFR mutation(s) and ii) tumors which are negative for E-cadherin staining and EGFR mutation.

Table IV. Gefitinib response summarized by the EGFR mutation status and by the staining of p-EGFR, p-Akt or E-cadherin.

| | EGFR mutation | | p-EGFR | | p-Akt | | E-cadherin | |
|---|---|---|---|---|---|---|---|---|
| | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| All patients | 10 | 14 | 3 | 21 | 3 | 21 | 19 | 5 |
| Responsive disease (CR + PR) | 6 | 2 | 1 | 7 | 1 | 7 | 6 | 2 |
| P | <0.005 | | 0.23 | | 0.23 | | 0.72 | |
| Controlled disease (CR + PR + SD) | 7 | 7 | 1 | 13 | 1 | 13 | 11 | 3 |
| P | <0.05 | | 0.54 | | 0.54 | | 0.95 | |

EGFR, epidermal growth factor receptor; p-EGFR, phosphorylated-EGFR; p-Akt, phosphorylated-Akt.

The current study warrants a larger one and presents an important question. We have six panels in Fig. 2, three of which showed significant differences and three of which did not. It is calculated that, if twice as many patients had been enrolled and had shown similar responsiveness and prognoses, all six sets of the two groups compared in Fig. 2 would have shown significant differences. To investigate this, a study should be scheduled where more than twice the number of patients is enrolled. We showed that tumors with a positive E-cadherin staining have a better prognosis after gefitinib therapy. It is, however, not clear whether the E-cadherin expression and EGFR mutation(s) contribute to it independently or synergistically. Basic and clinical researches addressing this issue may provide important information on the role of E-cadherin and EGFR in carcinogenesis.

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# Interferon-β augments eosinophil adhesion-inducing activity of endothelial cells

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ABSTRACT: **Viral infections induce exacerbations of asthma. One of the earliest host responses to viral infections is the production of innate cytokines including type I interferons (IFNs), such as IFN-β, which may act to modify airway inflammation. The objective of the present study was to investigate whether IFN-β modifies the eosinophil adhesion-inducing activity of endothelial cells.**

**Human umbilical vein endothelial cells (HUVECs) were stimulated with IFN-β for 24 h in the presence or absence of tumour necrosis factor (TNF)-α. Eosinophils were isolated from the peripheral blood of healthy volunteers. The ability of the IFN-β-stimulated HUVEC monolayers to induce eosinophil adhesion was assessed according to the eosinophil peroxidase assay.**

**Eosinophil adhesion to HUVECs was significantly augmented by IFN-β in the presence of TNF-α but not in its absence. The augmented adhesion was inhibited by anti-$\alpha_4$ integrin monoclonal antibody (mAb) or anti-$\beta_2$ integrin mAb. IFN-β significantly enhanced the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 on HUVECs in the presence of TNF-α.**

**Interferon-β can augment the adhesiveness of endothelial cells to eosinophils, mainly through the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. This action of interferon-β may contribute to the intensification of airway inflammation in asthma that is associated with exacerbations induced by viral infections.**

KEYWORDS: **Asthma, endothelial cells, eosinophilic airway inflammation, viral infection**

Acute respiratory infections commonly precede asthma exacerbations in both children and adults [1–3]. The majority of episodic exacerbations of asthma are induced by viral respiratory infections, in particular rhinovirus infections [4]. The mechanism by which viral respiratory infections exacerbate asthma is a complex process that may be regulated by the enhanced production of cytokines, chemokines and other classes of inflammatory molecules [4, 5]. An effective antiviral immune response requires the early clearance of viruses and the appropriate termination thereof, to minimise concomitant immunopathology and tissue damage. One of the earliest host responses to viral infections is the production of initial innate cytokines. These cytokines include type I interferons (IFNs) such as IFN-β [6, 7]. WARK *et al.* [7] recently reported that respiratory epithelial cells from asthmatics have a lower IFN-β-producing ability that is associated with a reduced ability to clear viruses. Since IFNs have a variety of pro-inflammatory actions on inflammatory cells, including eosinophils, epithelial cells and endothelial cells [8–12], it is theoretically conceivable that these cytokines may modify and aggravate the inflammatory status of airway diseases, including asthma, during or after viral infection.

Eosinophils are inflammatory cells predominantly found in the airways of asthmatic patients and are likely to contribute to the pathogenesis of asthma through the production of a variety of mediators including cysteinyl (cys) leukotriene (LT) and transforming growth factor-β [11–14]. Although neutrophils play central roles in asthma exacerbations induced by viral respiratory infections, clinical data support the involvement of eosinophils in virus-induced exacerbations and increased airway hyperresponsiveness in asthmatic patients [15–17]. In atopic asthmatics, for example, experimental infections with rhinovirus (RV)16 increased epithelial eosinophil counts; this increase appeared to persist up to convalescence [15]. In asthmatic patients with confirmed viral infection, sputum showed high eosinophilic cationic protein (ECP) levels [16]. In atopic mild asthmatics, increased airway hyperresponsiveness to histamine was correlated significantly with an increase in ECP levels and with changes in eosinophil levels in induced

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sputum after nasal administration of RV16 [17]. For eosinophils, the initial step in their participation in the airway inflammation of asthma is the adhesion of circulating eosinophils to vascular endothelial cells. It is generally accepted that this process is mainly mediated by the interaction between eosinophil integrin adhesion molecules (including $\alpha_4$ integrins, such as $\alpha_4\beta_1$ (also known as CD49d, CD29 or very late activation antigen-4), and $\beta_2$ integrins, such as $\alpha_L\beta_2$ (CD11a/CD18/lymphocyte function-associated antigen-1) and $\alpha_M\beta_2$ (CD11b/CD18/macrophage-1 antigen)) and their counter ligands (vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1) on endothelial cells [18, 19].

The objective of the present study was to evaluate whether IFN-β modifies the adhesive interaction between eosinophils and endothelial cells.

## MATERIAL AND METHODS

### Reagents

Percoll® was obtained from Pharmacia (Uppsala, Sweden). Anti-CD16 antibody-coated magnetic beads were purchased from Miltenyl Biotec (Auburn, CA, USA). Human umbilical vein endothelial cells (HUVECs) and HuMedia EG were purchased from Kurabo Industries Ltd (Osaka, Japan). Endothelial cell growth medium was purchased from Clonetics Corporation (Palo Alto, CA, USA). Hanks' balanced salt solution (HBSS), PBS and newborn calf serum (NCS) were obtained from Life Technologies (Grand Island, NY, USA). Foetal bovine serum was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). Recombinant human (rh-) IFN-α, IFN-β, IFN-γ and tumour necrosis factor (TNF)-α were purchased from R&D Systems (Minneapolis, MN, USA). Anti-$\alpha_4$-integrin monoclonal antibody (mAb; clone HP2/1) was purchased from Cosmo Bio Co. Ltd (Tokyo, Japan). Anti-$\beta_2$-integrin mAb (clone L130) was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Murine immunoglobulin (Ig)G1 was purchased from ICN Biomedicals, Inc. Anti-P-selectin glycoprotein ligand (PSGL)-1 (CD162) mAb (clone PL-1) was purchased from Immunotech, a Coulter company (Marseille, France). Other reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise stated.

### Preparation of HUVECs

HUVECs were prepared as previously described [20]. Briefly, HUVECs were incubated on type IV collagen-coated tissue culture flasks until confluent, transferred to collagen-coated 96-well tissue culture plates and then stimulated either with a combination of IFN-β (30–1,000 pM) and TNF-α (10 pM) or with IFN-β (30–1,000 pM) alone in 5% $CO_2$ at 37°C for 24 h. After incubation, the incubated mixture was decanted and the HUVECs were washed three times with HBSS. In selected experiments, HUVECs were fixed with 100 μL of 1% paraformaldehyde in PBS at room temperature for 15 min, in order to block the synthesis of mediators. After washing three times in HBSS, 200 μL of 1% glycine in HBSS were added and incubated at ambient temperature for 1 h to quench any residual paraformaldehyde. The plates were then decanted and washed three times in HBSS before use.

### Eosinophil isolation

Eosinophil isolation was performed according to the negative immunomagnetic bead selection method, as previously described [21]. To complete all the experiments, eosinophils were isolated from the peripheral blood of 42 healthy volunteers who were aged 20–29 yrs and had an equal sex distribution. Briefly, the heparinised blood was diluted with HBSS without $Ca^{2+}$ and then centrifuged on Percoll® (1.090 $g \cdot mL^{-1}$; 700 × $g$ for 20 min). Plasma, mononuclear cell bands and Percoll® were removed, and red blood cells in the pellets were lysed by hypotonic shock. Granulocytes obtained were washed in 4°C HBSS supplemented with 2% NCS and then incubated with anti-CD16 antibody-coated magnetic beads at 4°C for 40 min. The cells were filtered through the steel wool column in a magnetic field (Miltenyl Biotec) to remove neutrophils bound to magnetic beads. CD16-negative eosinophils (>98% purity and >99% viability) were collected, washed and then resuspended in HBSS supplemented with 0.1% gelatin (HBSS/0.1% gelatin).

### Eosinophil adhesion

Eosinophil adhesion to HUVECs was assessed by the residual eosinophil peroxidase activity of adherent eosinophils, as previously described [20–23]. Briefly, eosinophils (100 μL of $1 \times 10^5$ cells·$mL^{-1}$ in HBSS/0.1% gelatin) were placed onto the HUVEC monolayers and then incubated at 37°C for 30 min. After five washes in 37°C HBSS, 100 μL of HBSS/0.1% gelatin were added to the reaction wells. As standards, 100 μL of serially diluted cell suspensions ($1 \times 10^3$, $3 \times 10^3$, $1 \times 10^4$, $3 \times 10^4$ and $1 \times 10^5$ cells·$mL^{-1}$) were added to the empty wells. The o-phenylenediamine (OPD) substrate (1 mM OPD, 1 mM $H_2O_2$ and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all the wells. After incubation at room temperature for 30 min, 50 μL of 4 M $H_2SO_4$ were added to stop the reaction and absorbance at 490 nm was determined. The percentage of eosinophil adhesion was calculated from the log dose–response curve. Eosinophil viability after incubation, which was determined by trypan blue dye exclusion, exceeded 98%.

### Determination of VCAM-1 and ICAM-1 expression on HUVECs

The expression of VCAM-1 and ICAM-1 was determined by cell ELISA, as previously reported [20, 24]. Briefly, the HUVEC monolayers were incubated in the 96-well tissue culture plates and then stimulated with either a combination of IFN-β (30–1,000 pM) and TNF-α (10 pM) or with IFN-β (30–1,000 pM) alone at 37°C for 24 h [20, 24]. Prior to the evaluation, HUVECs were washed and incubated at 37°C for 30 min with a blocking buffer (PBS containing 5% NCS and 3% nonfat dry milk). Primary antibodies (obtained from R&D Systems), *i.e.* anti-ICAM-1 mAb (clone BBIG-I1), anti-VCAM-1 mAb (clone BBIG-V1) and isotype-matched control murine IgG1, were added to the wells and the incubation was then resumed at 37°C for a further 2 h. HUVECs were washed three times in the blocking buffer and secondary antibody (peroxidase-conjugated sheep anti-murine IgG) was added to the wells. Following a 2-h incubation, cells were washed three times in PBS. The peroxidase conjugate was detected using the OPD substrate in the citrate-urea buffer according to a procedure similar to that used in the eosinophil adhesion assay. The VCAM-1 or ICAM-1 concentration in cells was expressed as absorbance at

490 nm and was reported as the actual value minus absorbance of isotype-matched control murine IgG1.

### *Statistical analysis*

Values are expressed as mean±SEM. Paired t-tests were conducted for comparison of two groups and repeated-measures ANOVA with Scheffé's constants was used to compare more than two groups. A p-value <0.05 was considered statistically significant.

## RESULTS

### *Effects of IFN-β on the adhesive interaction between eosinophils and endothelial cells*

An initial series of experiments was conducted to determine whether IFN-β directly modifies the adhesiveness of eosinophils. Eosinophils were stimulated with rh-IFN-β (30–1,000 pM) and their adhesiveness to HUVECs was examined. The adhesiveness was not modified by IFN-β (n=5, data not shown). Subsequently, the ability of IFN-β to modify the adhesiveness of endothelial cells to eosinophils was evaluated in the presence or absence of TNF-α. HUVECs were stimulated with either IFN-β alone (30–1,000 pM) or with a combination of IFN-β (30–1,000 pM) and TNF-α (10 pM) in 5% $CO_2$ at 37°C for 24 h, and eosinophil adhesion to HUVECs was then examined. The percentages of eosinophil adhesion to resting and TNF-α (10 pM)-stimulated HUVECs with no IFN-β were 2.8±1.2 and 6.6±1.8, respectively; as expected, eosinophil adhesion to HUVECs was augmented by TNF-α stimulation ($p<0.01$, n=8). IFN-β (30–1,000 pM) stimulation alone did not modify the adhesiveness of HUVECs to eosinophils (n=8; fig. 1a). However, IFN-β (300–1,000 pM) stimulation significantly upregulated the eosinophil adhesion-inducing ability of HUVECs in the presence of TNF-α (adhesion of eosinophils: 6.6±1.8% by control *versus* 14.7±3.5% by 300 pM ($p<0.01$) and 14.8±3.1% by 1000 pM ($p<0.001$); n=8; fig. 1b). The IFN-β (300 pM)-augmented adhesiveness in TNF-α (10 pM)-stimulated HUVECs was not observed in the presence of anti-IFN-β mAb (adhesion of eosinophils in the presence of isotype-matched control mouse IgG1: 7.2±2.5% by control *versus* 11.4±3.5% by 300 pM IFN-β ($p<0.01$); in the presence of anti-IFN-β mAb: 7.8±3.1% by control *versus* 8.6±3.2% by 300 pM IFN-β (p=nonsignificant (NS)); n=5). Eosinophil adhesion to resting or TNF-α (10 pM)-stimulated HUVECs was not modified by anti-IFN-β mAb (data not shown).

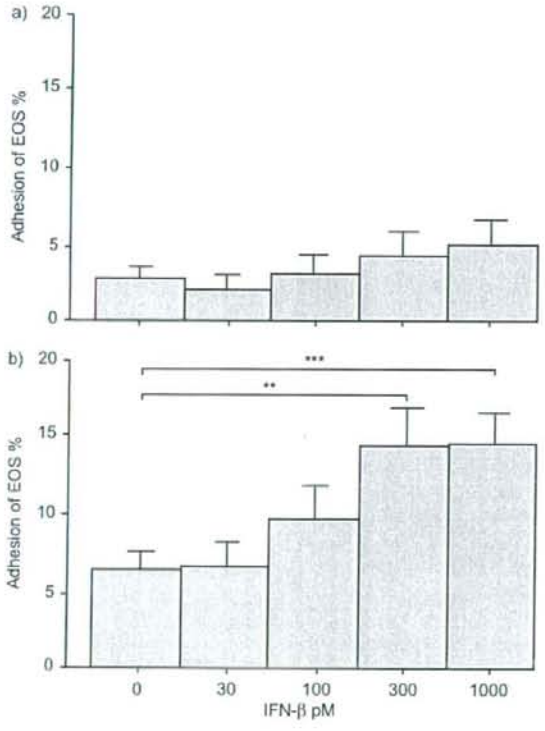


FIGURE 1. Effects of interferon (IFN)-β on the eosinophil (EOS) adhesion-inducing ability of human umbilical vein endothelial cells in the a) absence or b) presence of tumour necrosis factor-α (10 pM). For each bar, the mean±SEM of eight experiments using EOS from different donors is shown. **: $p<0.01$; ***: $p<0.001$.

The effects of incubation time of IFN-β on the enhanced adhesiveness of TNF-α (10 pM)-stimulated HUVECs were then evaluated. TNF-α (10 pM)-stimulated HUVECs were incubated in the presence or absence of IFN-β (300 pM) for a range of incubation times (1–24 h) and then washed, and HUVEC adhesiveness to eosinophils was evaluated. The present study revealed that the IFN-β (300 pM)-augmented adhesiveness of HUVECs to eosinophils appeared at 4 h and lasted until 24 h of incubation time ($p<0.05$ for comparison of each time-point with the previous one; n=5; fig. 2).

To examine whether HUVECs by themselves generate IFN-β, HUVECs were cultured in the presence or absence of TNF-α (10 pM) for 24 h and then the supernanatants were evaluated by ELISA. IFN-β protein was not detected in the supernatants of these HUVECs.

### *Effects of anti-adhesion molecule antibodies on the eosinophil adhesion augmented by IFN-β*

To identify eosinophil adhesion molecules that are involved in the IFN-β-augmented adhesion of eosinophils to HUVECs, eosinophils were pretreated with either anti-$\beta_2$ integrin mAb (clone L130, mouse IgG1, 3 $\mu g \cdot mL^{-1}$), anti-$\alpha_4$ integrin mAb (clone HP2/1, mouse IgG1, 3 $\mu g \cdot mL^{-1}$), anti-PSGL-1 (CD162) mAb (clone PL-1, mouse IgG1, 3 $\mu g \cdot mL^{-1}$) or isotype-matched control murine IgG1 (3 $\mu g \cdot mL^{-1}$) at ambient temperature for 15 min. Subsequently, eosinophil adhesion to HUVECs was stimulated with a combination of IFN-β (300 pM) plus TNF-α (10 pM) or with TNF-α (10 pM) alone. IFN-β (300 pM) stimulation significantly augmented adhesiveness of HUVECs to eosinophils when eosinophils were pretreated with murine IgG1 or anti-PSGL-1 mAb ($p<0.01$; n=5; fig. 3). In contrast, the effects of IFN-β (300 pM) on the augmented adhesion of eosinophils to HUVECs were significantly inhibited by either anti-$\beta_2$ integrin mAb or anti-$\alpha_4$ integrin mAb (fig. 3), suggesting roles for the counter ligands of these integrins, namely ICAM-1 and VCAM-1. When anti-$\beta_2$ integrin mAb was present, the augmentation of eosinophil adhesion to HUVECs by IFN-β (300 pM) stimulation was modest but remained significant (1.8±0.5% by TNF-α (10 pM) alone *versus*

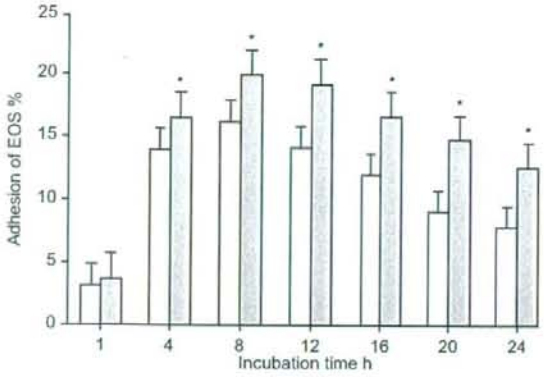


FIGURE 2. Effects of incubation time (1–24 h) of tumour necrosis factor-α (10 pM) in the presence (▪) or absence (□) of interferon-β (300 pM) on the eosinophil (EOS) adhesion-inducing ability of human umbilical vein endothelial cells. For each bar, the mean±SEM of five experiments using EOS from different donors is shown. *: $p<0.05$ for comparison with previous time-point.

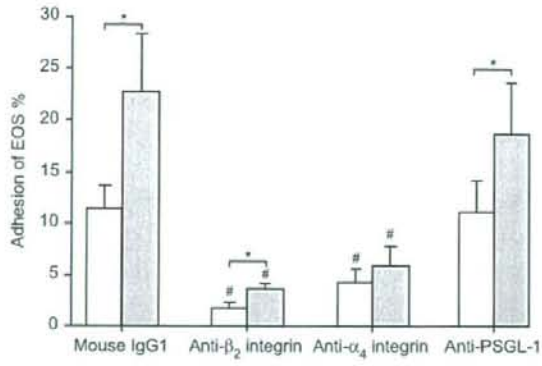


FIGURE 3. Effects of anti-adhesion molecule antibodies on eosinophil (EOS) adhesion to human umbilical vein endothelial cells stimulated with tumour necrosis factor-α (10 pM) in the presence (▪) or absence (□) of interferon (IFN)-β (300 pM). For each bar, the mean±SEM of seven experiments using EOS from different donors is shown. Ig: immunoglobulin; PSGL: P-selectin glycoprotein ligand. *: $p<0.05$ *versus* no IFN-β, #: $p<0.05$ *versus* murine IgG1.

3.6±0.8% by TNF-α (10 pM) plus IFN-β (300 pM); $p=0.03$; $n=7$; fig. 3). Conversely, the augmented adhesiveness of HUVECs to eosinophils by IFN-β (300 pM) stimulation was not significant when eosinophils were pretreated with anti-$\alpha_4$ integrin mAb (4.4±1.1% by TNF-α (10 pM) alone *versus* 5.9±1.4% by TNF-α (10 pM) plus IFN-β (300 pM); $p>0.05$; $n=7$).

### *Effects of IFN-β on the expression of endothelial cell adhesion molecules*

Whether IFN-β modifies the expression of ICAM-1 and VCAM-1 on HUVECs was next evaluated. HUVECs were stimulated with IFN-β (30–1,000 pM) in the presence or absence of TNF-α (10 pM) for 24 h; the expression of ICAM-1 and VCAM-1 was then determined by cell ELISA. In the absence of TNF-α, IFN-β (30–1,000 pM) modified the expression of neither VCAM-1 (fig. 4a) nor ICAM-1 (fig. 4b). Conversely, IFN-β (300–1,000 pM) significantly upregulated the expression of VCAM-1 on HUVECs stimulated with TNF-α (10 pM) (absorbance optical density (OD): 0.026±0.003 by TNF-α alone *versus* 0.072±0.012 by TNF-α plus IFN-β (300 pM); $p=0.03$; $n=6$; fig. 4a). Similarly, IFN-β (30–1,000 pM) significantly upregulated the expression of ICAM-1 on HUVECs stimulated with TNF-α (OD: 0.492±0.023 by TNF-α alone *versus* 0.614±0.019 by TNF-α (10 pM) plus IFN-β (30 pM); $p<0.01$; $n=6$; fig. 4b). Finally, the effects of IFN-β on the expression of VCAM-1 and ICAM-1 on TNF-α-stimulated HUVECs were also confirmed by flow cytometric analysis. Following stimulation with 300 pM IFN-β for 24 h, the expression of both VCAM-1 and ICAM-1 on HUVECs stimulated with TNF-α were enhanced (mean fluorescence index for VCAM-1: 24.4±8.5 by medium control *versus* 42.7±16.5 by IFN-β ($p<0.01$); for ICAM-1: 708.7±108.2 by medium control *versus* 1,450.7±144.5 by IFN-β ($p<0.01$); $n=4$).

### *Mechanism of IFN-β-augmented adhesion of eosinophils to endothelial cells*

IFN-β may trigger the production of pro-inflammatory mediators from endothelial cells and, hence, may augment eosinophil adhesiveness. To corroborate this hypothesis, HUVECs were stimulated with or without IFN-β (300 pM) in the presence of TNF-α (10 pM) for 24 h, washed, and then fixed with 1% paraformaldehyde. Following washing, HUVECs were used to evaluate eosinophil adhesiveness. Even following the fixation, HUVEC adhesiveness to eosinophils augmented by the addition of IFN-β was significant (10.3±5.1% by TNF-α alone *versus* 14.2±5.3% by TNF-α plus IFN-β; $p<0.05$; $n=6$). Eosinophils may be activated by the interaction with VCAM-1 or ICAM-1 on HUVECs that were stimulated with IFN-β, and may thus augment their adhesiveness in an autocrine/paracrine fashion. For example, eosinophils are capable of producing cysLT, which can enhance the adhesiveness of eosinophils themselves. To assess this possibility, eosinophils were pretreated with a cysLT receptor antagonist, montelukast (1 μM), and eosinophil adhesiveness to HUVECs stimulated with or without IFN-β in the presence of TNF-α was evaluated. The cysLT antagonist did not attenuate eosinophil adhesiveness to HUVECs stimulated with or without IFN-β in the presence of TNF-α (without IFN-β (TNF-α alone): 6.6±2.1 *versus* 7.5±1.5% for control *versus* montelukast (p=NS); with IFN-β: 10.5±1.5 *versus* 11.9±2.1% for control *versus* montelukast (p=NS); $n=4$; data not shown).

### *Effects of a variety of IFNs on endothelial cell adhesiveness to eosinophils*

To evaluate whether the adhesiveness of endothelial cells to eosinophils is modified by other classes of IFNs, HUVECs were treated with IFN-α, a type I IFN, or with IFN-γ, a type II IFN, in the presence or absence of TNF-α (10 pM) at 37°C for 24 h. The ability to induce increased eosinophil adhesion of HUVECs was thus examined. None of the IFNs (α, β or γ) at 300 pM modified HUVEC adhesiveness to eosinophils in the absence of TNF-α (10 pM). However, IFN-α, IFN-β and IFN-γ (all 300 pM) significantly augmented the eosinophil adhesion-inducing ability of HUVECs in the presence of TNF-α (10 pM; 5.2±0.3% by control *versus* 12.7±2.5% by IFN-α ($p<0.05$),

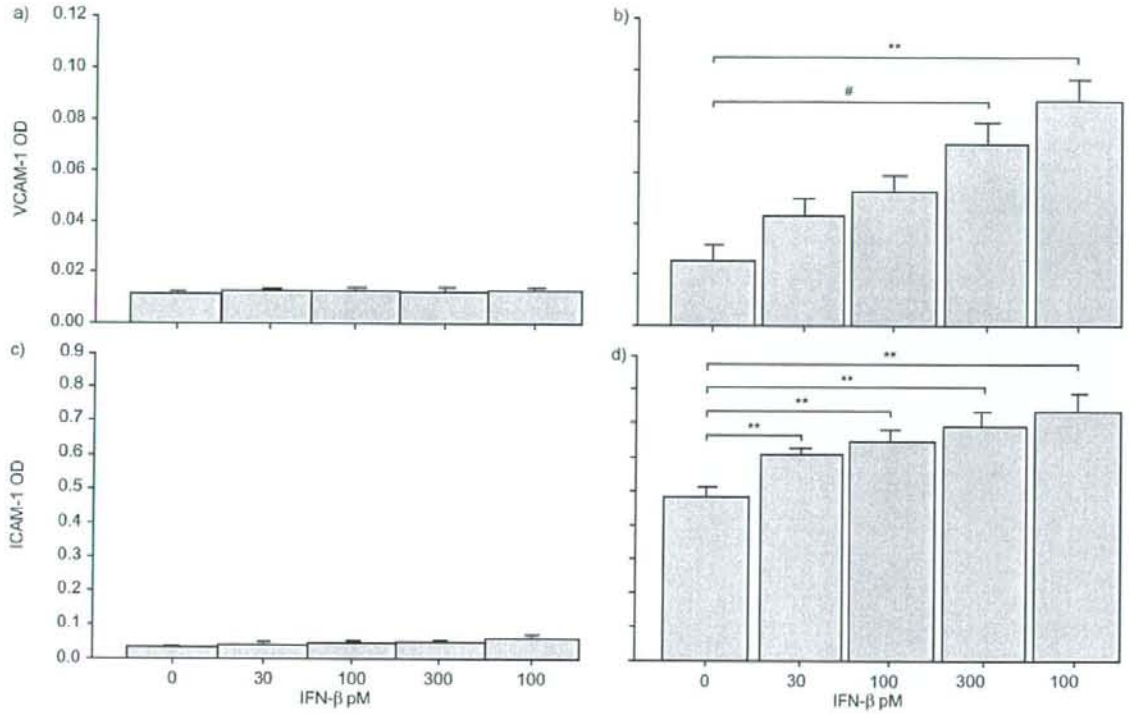


FIGURE 4. Effects of interferon (IFN)-β on the expression of vascular cell adhesion molecule (VCAM)-1 (a and b) and intercellular adhesion molecule (ICAM)-1 (c and d) on human umbilical vein endothelial cells in the absence (a and c) or presence (b and d) of tumour necrosis factor-α (10 pM). For each bar, the mean±SEM of six different experiments is shown. OD: optical density. **: $p<0.01$; #: $p=0.03$.

13.7±2.8% by IFN-β ($p<0.05$) and 12.1±2.1% by IFN-γ ($p<0.05$); n=4; fig. 5b). There was no significant difference between the three IFN-treated groups. Finally, IFN-α, IFN-β and IFN-γ (all 300 pM) significantly augmented the expression of VCAM-1 and ICAM-1 on HUVECs in the presence of TNF-α (10 pM) and there was no significant difference between the three groups ($p<0.01$; n=4; fig. 5c–f).

## DISCUSSION

In the present study, evidence was provided that the IFN family, including IFN-β, augments the adhesiveness of endothelial cells to eosinophils, which may be a novel regulatory mechanism for eosinophilic inflammation in the airways of patients with asthma. Although IFN-β by itself did not directly modify eosinophil adhesiveness, it was observed that endothelial cell stimulation with IFN-β augments the eosinophil adhesion-inducing ability of HUVECs in the presence of TNF-α. The neutralising effects of mAbs on IFN-β demonstrated that the augmented adhesiveness of endothelial cells to eosinophils was mediated specifically by this cytokine. The effect of IFN-β appears to involve the expression of adhesion molecules on endothelial cells, a conclusion that can be drawn from the following results. First, the adhesiveness of eosinophils augmented by IFN-β stimulation was blocked by anti-$\alpha_4$ integrin or anti-$\beta_2$ integrin antibody. Secondly, IFN-β significantly augmented the expression of VCAM-1 and ICAM-1. Although both endothelial cells and eosinophils could produce mediators which may modify eosinophil adhesiveness, the effects of IFN-β were observed in fixed HUVECs or eosinophils treated with inhibitors for representative eosinophil-derived mediators. Therefore, the current authors speculate that IFN-β mainly enhances the expression of VCAM-1 and ICAM-1, and that the effect also confers greater adhesiveness to eosinophils. Finally, the present study provided evidence that not only IFN-β but also IFN-α and IFN-γ augment the adhesiveness of endothelial cells to eosinophils. Collectively, the current results suggest a potentially important biological effect of IFNs in the development of adhesive interaction between eosinophils and endothelial cells when IFNs and TNF-α are overproduced in the airways.

The IFN family of cytokines has essential roles in immunity. There is evidence that the IFN family modifies either the expression of adhesion molecules or the proliferation of endothelial cells. The present study is the first to verify that IFNs actually augment the adhesiveness of endothelial cells to eosinophils. This observation is in agreement with previous studies on the interaction between IFN-β and human vascular endothelial cells. For example, MILLER *et al.* [25] reported that IFN-β, but not IFN-γ, modestly enhances the expression of

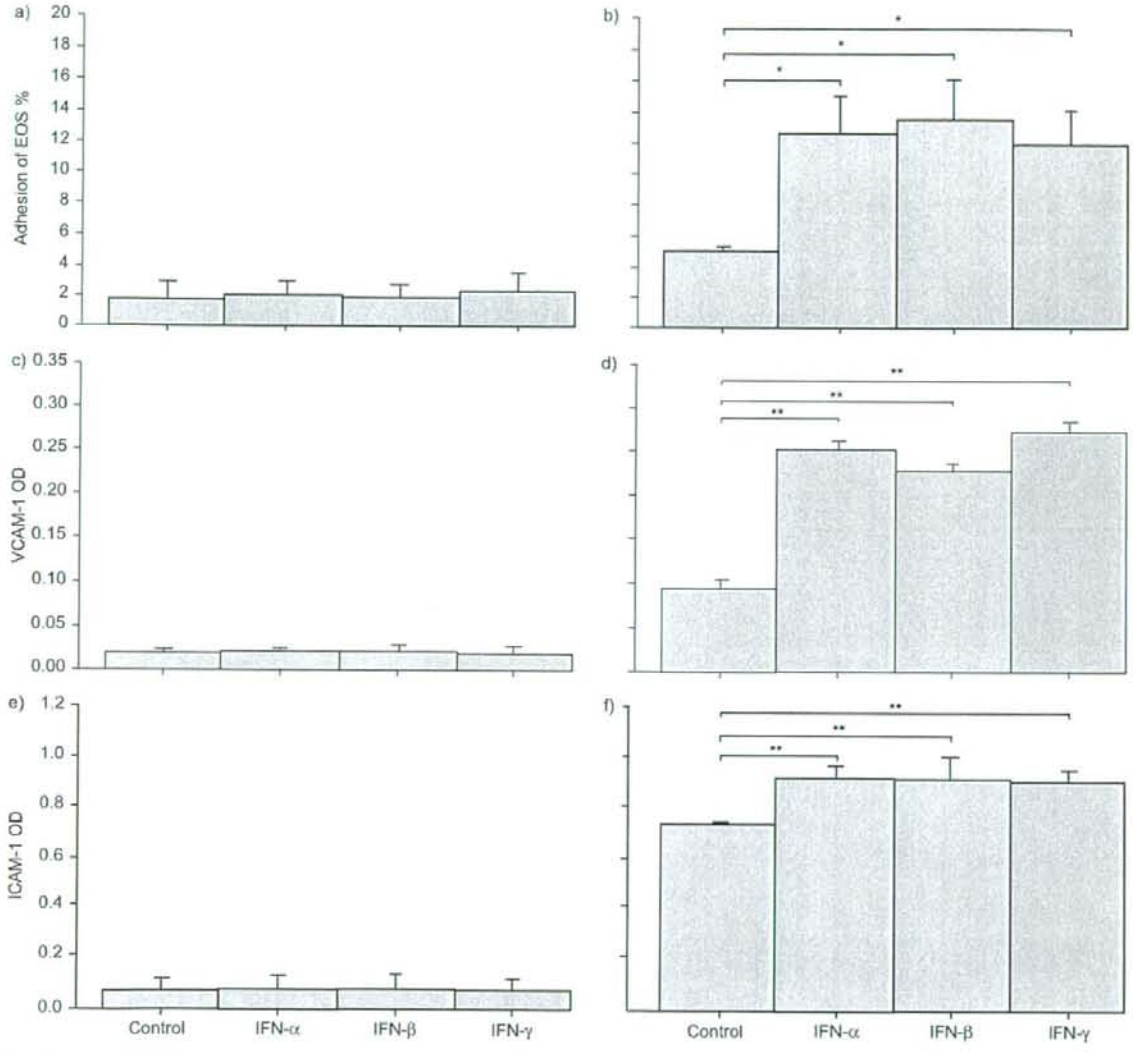


FIGURE 5. Effects of interferon (IFN)-α, IFN-β and IFN-γ (all 300 pM) on the eosinophil (EOS) adhesion-inducing ability (a and b) and the expression of vascular cell adhesion molecule (VCAM)-1 (c and d) and intercellular adhesion molecule (ICAM)-1 (e and f) of human umbilical vein endothelial cells in the absence (a, c and e) or presence (b, d and f) of tumour necrosis factor-α (10 pM). For each bar, the mean±SEM of four different experiments is shown. OD: optical density. *: $p<0.05$; **: $p<0.01$.

ICAM-1. Similarly, LECHLEITNER *et al.* [11] reported that IFN-α and IFN-γ enhance the TNF-α-induced transcription of VCAM-1 mRNA and the protein expression in human endothelial cells at the transcriptional level *via* the nuclear IFN-related factor-1-dependent pathway. KITAYAMA *et al.* [26] reported that the culture supernatants of HUVEC stimulated with TNF-α and IFN-γ induced eosinophil chemotaxis and eosinophil adhesion to ICAM-1 and VCAM-1 mainly *via* generation of CCR3 ligands. More recently, GOMEZ and REICH [27] provided evidence that IFNs can stimulate the proliferation of primary human endothelial cells and that the effect may be attributed to the activation of signal transducer and activator of transcription (STAT)3 and STAT5. These reports and the present observations suggest that the IFN family augments either activation status or adhesiveness of endothelial cells and, in turn, contributes to the development of eosinophilic inflammation in the airways of patients with allergic diseases such as asthma.

For eosinophils to adhere and then migrate across endothelial cells, endothelial cell adhesion molecules are required. In the present study, it was observed that the IFN-β-augmented adhesiveness of HUVECs to eosinophils in the presence of TNF-α was inhibited by either anti-$\alpha_4$ integrin mAb or anti-$\beta_2$

integrin mAb (mAbs against counter ligands for VCAM-1 and ICAM-1, respectively). Although the expression of both VCAM-1 and ICAM-1 was augmented by IFN-β in the presence of TNF-α, the effects of IFN-β (300–1,000 pM) on VCAM-1 were consistent with its effects on HUVEC adhesiveness to eosinophils (figs 1 and 4a). Meanwhile, the enhanced expression of ICAM-1 was observed with lower concentrations of IFN-β (⩾30 pM; fig. 4b). The ability to induce eosinophil spontaneous adhesion is more potent with VCAM-1 than with ICAM-1 [28]. From this point of view, the current results demonstrated that the IFN-β-augmented adhesion of eosinophils to HUVECs was still observed following treatment with anti-$\beta_2$ integrin mAb. Conversely, the augmented adhesion of eosinophils was abrogated by anti-$\alpha_4$ integrin mAb. Therefore, it can be speculated that VCAM-1 takes precedence in the induction of the enhanced eosinophil adhesion in this system.

Viral respiratory infections can cause bronchial hyperresponsiveness and exacerbate asthma. In general, neutrophils play major roles in asthma exacerbations induced by viral infections. However, eosinophilic inflammation can also be enhanced under certain conditions. Clinical data support the possible involvement of eosinophils in virus-induced exacerbations and increased airway hyperresponsiveness in asthmatic patients: experimental infections with RV16 led to increases in eosinophils and ECP levels in the airways and to airway hyperresponsiveness in atopic asthmatics [15–17]. In infants with respiratory syncytial virus (RSV) bronchiolitis, ECP and $LTC_4$ levels in upper airway secretions are significantly associated [29]. In a guinea pig model of asthma, both airway responsiveness and eosinophil accumulation in the airways increased after a respiratory infection with parainfluenza-3 virus [30]. In mice, RSV infection, which induces an immune response dominated by IFN-γ, resulted in airway hyperresponsiveness and eosinophil influx into the airways [31].

WARK *et al.* [7] reported that respiratory epithelial cells from asthmatics produce lower levels of IFN-β. They demonstrated that both impaired apoptosis and increased virus replication in infected asthmatic cells are recovered by exogenous IFN-β, suggesting a possible use for type I IFNs in the treatment of virus-induced asthma exacerbations. O'SULLIVAN *et al.* [32] reported that treatment with inhalational corticosteroids reduced the number of cells expressing IFN-β in the lamina propria of bronchial biopsy specimens obtained from mild asthmatics. These findings suggest that endothelial cells in the airways of asthma patients may be exposed to relatively lower concentrations of IFN-β in asthma. In the present study, however, the authors focused on a pro-inflammatory aspect of IFN-β and a possible role of this cytokine in asthma exacerbation. Despite its important role in anti-viral immunity, IFN-β may enhance airway inflammation *via* an enhancement of eosinophil adherence to endothelial cells. This effect of IFN-β may be important in a variety of clinical conditions seen with asthma. For example, in cases of cigarette smoking, production of IFN-β by the airway leukocytes from corticosteroid-treated asthmatics is enhanced [33]. RODEL *et al.* [34] demonstrated that *Chlamydia pneumoniae* induced the production of IFN-β in bronchial and vascular smooth muscle cells in the presence of TNF-α. In this context, TLIBA *et al.* [35] reported that a combination of TNF-α and IFN-β acts synergistically to induce CD38 mRNA and protein expression in human smooth muscle cells. These two studies are consistent with the present study with respect to the interaction between IFN-β and TNF-α. At present, there is not enough clinical evidence that IFN-β is actually involved in asthma exacerbation. However, the current authors speculate that such an effect of IFN-β on the interaction between endothelial cells and eosinophils may be one mechanism for the enhanced airway inflammation seen with asthma exacerbation.

The current results provide new insights into the mechanisms that regulate eosinophilic inflammation in the airways of asthmatic patients, especially those with viral infections. When activated, a variety of cells, including T-helper type 1 cells, epithelial cells and natural killer cells, are capable of producing IFNs at the sites of airway inflammation. Therefore, endothelial cells are likely to be exposed to IFNs, which enhance their interaction with eosinophils at least partially through the enhancement of VCAM-1 or ICAM-1 expression. Interaction with VCAM-1 or ICAM-1 may enhance the effector functions of eosinophils, *e.g.* the release of radical oxygen species and the production of cysLTs [25, 27]. The exposure of eosinophils to these products in an autocrine and/or paracrine fashion possibly modifies their functions. For example, a chemotactic response and the interaction between ICAM-1 and eosinophils would be augmented by newly produced cysLTs [21]. An oxygen metabolite, hydrogen peroxide, further augments eosinophil adhesiveness to ICAM-1 [22]. Hence, the present study demonstrated that IFN-β and other IFNs can augment the adhesive interaction between eosinophils and endothelial cells, with the resultant modification of other adhesion-dependent effector functions of eosinophils through their interaction with either VCAM-1 or ICAM-1. These changes may contribute to the eventual manifestation of airway inflammation in asthmatic patients. Understanding the clinical relevance of the effects mediated by IFN-β may have important implications in designing therapeutic strategies for asthmatic patients with viral respiratory infection-induced exacerbations.

### *Conclusion*

Interferon-β can augment the eosinophil adhesion-inducing activity of endothelial cells in the presence of tumour necrosis factor-α, mainly through the enhancement of vascular cell adhesion molecule-1 or intercellular adhesion molecule-1 expression. This action of interferon-β could, in turn, potentially contribute to the intensification of airway inflammation in asthmatic patients that is associated with exacerbations induced by viral infections.

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Review

# The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease

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**Abstract**

The production of a variety of lipid mediators is enhanced in bone-resorptive diseases such as osteoporosis, rheumatoid arthritis, osteoarthritis, and periodontitis. Prostaglandin $E_2$ ($PGE_2$) is one of the most notable lipid mediators of bone remodeling, and has been linked clinically to many bone-resorptive diseases. *In vitro* studies with bone cell cultures have demonstrated that the bone-resorptive activity of $PGE_2$, which is mediated by receptor activator of NF-κB ligand (RANKL), is key for the induction of osteoclast formation. Furthermore, interleukin (IL)-1- and IL-6-stimulated bone resorption involves $PGE_2$ production. In addition to its bone-resorptive effects, $PGE_2$ promotes bone formation *in vitro* by stimulating osteoblastic proliferation and differentiation. The multifaceted nature of $PGE_2$ makes it difficult to discern its role during bone remodeling. Leukotrienes (LTs), and particularly $LTB_4$, have also been implicated in bone remodeling and disease—specifically in rheumatoid arthritis. Moreover, recent studies from our laboratory have shown that platelet-activating factor (PAF) receptor-deficient mice develop only mild osteoporosis. Osteoclast survival in these mice is shortened and osteoclastic bone resorption is impaired. This review article focuses on these families of lipids and their function during bone metabolism and disease.


*Keywords:* $PGE_2$; Osteoblast; Osteoclast; Osteoporosis; PAF

*Abbreviations:* ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; ALP, alkaline phosphatase; BLT, LTB4 receptor; BMP, bone morphogenetic protein; CAIA, collagen antibody-induced arthritis; cAMP, cyclic AMP; CIA, collagen-induced arthritis; COX, cyclooxygenase; cPGES, cytosolic PGES; $cPLA_2$, cytosolic $PLA_2$; CRTH2, chemoattractant receptor-homologous molecule expressed on TH2 cells; CysLT, cysteinyl LT receptor; DHA, docosahexaenoic acid; DP, $PGD_2$ receptor; EP, $PGE_2$ receptor; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FLAP, 5-LO-activating protein; GPCR, G protein-coupled receptor; Gs, stimulatory G protein; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; IL, interleukin; IL-6R, IL-6 receptor; IP, $PGI_2$ receptor; 5-LO, 5-lipoxygenase; LPA, lysophosphatidic acid; LPS, lipopolysaccharide; LT, leukotriene; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MMPs, matrix metalloproteinases; mPGES, membrane-associated PGES; NSAID, nonsteroidal anti-inflammatory drug; OPG, osteoprotegerin; OXE, oxoeicosanoid receptor; 5-oxo-ETE, 5-oxo-eicosatetraenoic acid; PAF, platelet-activating factor; PAFR, PAF receptor; PAFR-KO, PAFR-knockout; PG, prostaglandin; PGES, PGE synthase; $PGI_2$, prostaglandin $I_2$; PKA, protein kinase A; $PLA_2$, phospholipase $A_2$; PPAR, peroxisome proliferator-activated receptor; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor-jB ligand; sIL-6R, soluble IL-6R; $sPLA_2$, secretory $PLA_2$; TNF, tumor necrosis factor; TX, thromboxane; VEGF, vascular endothelial growth factor.

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**Contents**



## 1. Introduction

Many lipids serve as signaling molecules, including prostanoids (prostaglandins (PGs) and thromboxanes (TXs)), leukotrienes (LTs), platelet-activating factor (PAF), sphingosine 1-phosphate, lysophosphatidic acid (LPA), and endocannabinoids. Several of these are produced by the hydrolytic action of phospholipase $A_2$ ($PLA_2$) enzymes on membrane glycerophospholipids (Fig. 1). To date, four distinct groups of $PLA_2$ enzymes have been identified [1,2]: a low molecular weight (14–17 kDa) secretory $PLA_2$ (s$PLA_2$) group, a high molecular weight cytosolic $PLA_2$ (c$PLA_2$) group, which includes the 85-kDa calcium-sensitive c$PLA_2\alpha$, a calcium-independent $PLA_2$ (i$PLA_2$) group, and a PAF acetylhydrolase group. Most of these $PLA_2$ isozymes release polyunsaturated fatty acids from the *sn*-2 position of glycerophospholipids. Among fatty acids, arachidonic acid is the most important molecule, because it is metabolized to prostanoids and LTs (Fig. 1). Among $PLA_2$ enzymes, c$PLA_2\alpha$ plays a dominant role in arachidonic acid release owing to two distinct characteristics: the tightly regulated activation by submicromolar calcium and phosphorylation in response to extracellular stimuli and the substrate preference for arachidonic acid-containing phospholipids over the others [3]. Cyclooxygenases (COX-1 [4–6] and COX-2 [7]) catalyze the cyclooxygenation of arachidonic acid to $PGG_2$ followed by the hydroperoxidation of $PGG_2$ to $PGH_2$. COX-1 is constitutively expressed and believed to maintain homeostatic conditions, while COX-2 is encoded by a stress-responding gene and responsible for the production of high levels of prostanoids during inflammation [8]. $PGH_2$ can then be converted into $PGE_2$ through the action of PGE synthase (PGES). Several PGES isozymes have been identified including membrane-associated PGES-1 (mPGES-1), whose expression is induced by proinflammatory stimuli [9,10], mPGES-2, and cytosolic PGES (cPGES) that are expressed constitutively [11,12]. The conversion of $PGH_2$ to the other bioactive products, including $PGD_2$, $PGF_2\alpha$, $PGI_2$ (prostacyclin), and $TxA_2$, via specific synthases is also biologically important [13].

In contrast to COX enzymes, 5-lipoxygenase (5-LO) is required for LT biosynthesis [14,15]. In conjunction with 5-LO-activating protein (FLAP) [16], 5-LO converts arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is then dehydrated to $LTA_4$ by the same enzyme [17,18]. 5-HpETE can also converted by peroxidase(s) to 5-hydroxyeicosatetraenoic acid (5-HETE), a precursor of 5-oxo-eicosatetraenoic acid (5-oxo-ETE) [19]. $LTA_4$ is hydrolyzed into bioactive $LTB_4$ by $LTA_4$ hydrolase [20,21] or is converted into $LTC_4$ by $LTC_4$ synthase [22]. $LTC_4$ is sequentially metabolized to $LTD_4$ and then to $LTE_4$ [23]. $LTC_4$, $LTD_4$, and $LTE_4$ are bioactive, and comprise cysteinyl leukotrienes, because they contain a cysteine residue.

PAF is synthesized by either the *de novo* and remodeling pathway (Fig. 2) [24,25]. The remodeling pathway is regu-

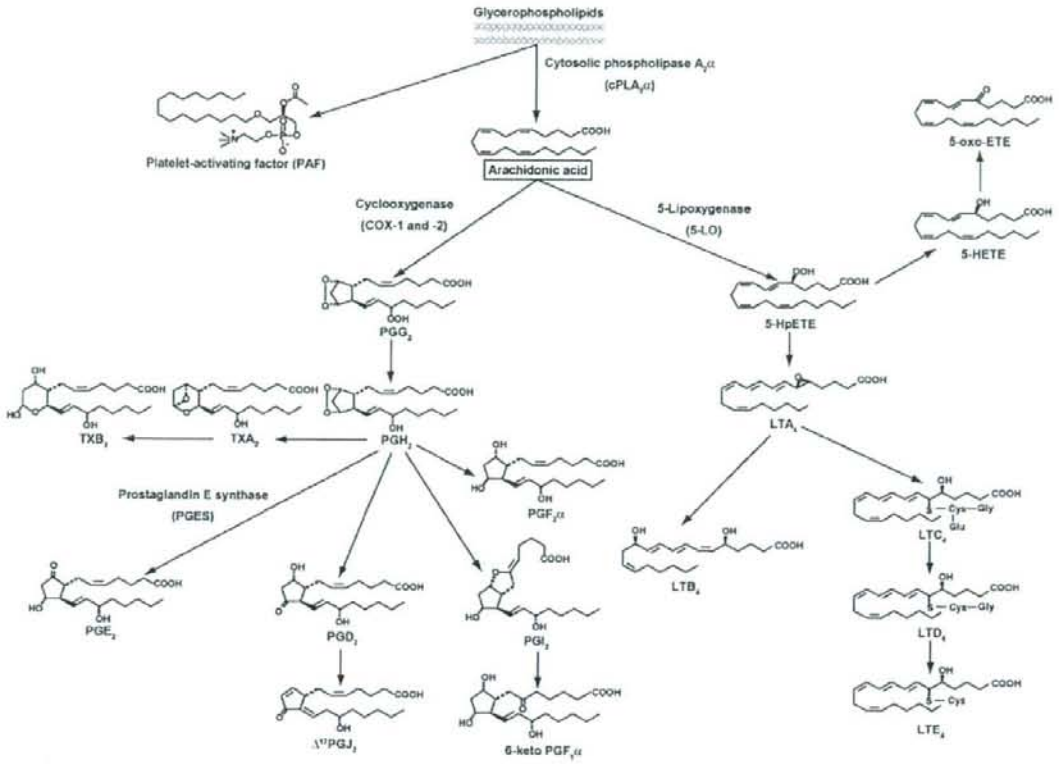


Fig. 1. Metabolic pathways for arachidonic acid. $PLA_2$ enzymes, particularly $cPLA_2\alpha$, release arachidonic acid from the *sn*-2 position of glycerophospholipids in biomembranes. Arachidonic acid is metabolized to prostanoids and LTs by COX and 5-LO, respectively. PAF is derived from the glycerophospholipids remaining after the release of arachidonic acid by $cPLA_2\alpha$. For the details of PAF synthesis reactions, see "the remodeling pathway" in Fig. 2.

lated by extracellular stimuli and is responsible for the bulk of the PAF synthesis under inflammatory conditions. Stimulus-coupled PAF biosynthesis is initiated by the activation of $PLA_2$ enzymes that hydrolyze 1-*O*-alkyl-phosphatidylcholine in biomembranes to 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) [24,25]. Like prostanoids and LTs, the action of $cPLA_2\alpha$ is important for this hydrolysis reaction [26,27]. Although lyso-PAF is biologically inactive, it becomes acetylated to form PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by acetyl-CoA:lyso-PAF acetyltransferase, which we have recently cloned [28]. The lipid mediators described above exert their bioactivities on a variety of cells through their specific receptors, which are mostly G protein-coupled receptors (GPCRs) [13,29,30].

Bone is a complex living tissue that has both protective and supportive functions while actively participating in calcium homeostasis. Bone tissues continually alter their internal structure by removing old bone and replacing it with newly formed bone, *i.e.*, bone remodeling, in which osteoblasts and osteoclasts are key players [31]. Osteoblasts arise from local osteoprogenitor cells and are responsible for the bone-matrix production. In addition, osteoblasts are also required for osteoclast formation. Osteoclasts, originating from hematopoietic tissues, are multinucleated cells that resorb bone. These two cell types participate in bone remodeling under the control of many systemic hormones and local regulators including parathyroid hormone (PTH) [32], 1α,25-dihydroxyvitamin $D_3$ (1α,25-$(OH)_2$ vitamin $D_3$) [33], glucocorticoids [34] and estrogen [35]. Fibroblast growth factor (FGF) [36], bone morphogenetic proteins [36], insulin-like growth factor [36], platelet-derived growth factor [36], and several cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 [37] are also known to regulate bone metabolism.

Many studies have examined the physiological and pathological effects of prostanoids on bone [38]. The bone-resorptive action of $PGE_2$ was first reported *in vitro* more than 30 years ago in a rat organ culture system that included both osteoblasts and osteoclasts [39]. Since then, the effects of $PGE_2$ on osteoblasts and osteoclasts have been revealed by both *in vitro* and *in vivo* studies. Currently, $PGE_2$ is recognized as one of the most important local regulators of bone metabolism. Therefore, we will focus this review article on the role of $PGE_2$ in this process. However, other prostanoids and LTs may also play a significant role in bone metabolism and their function will

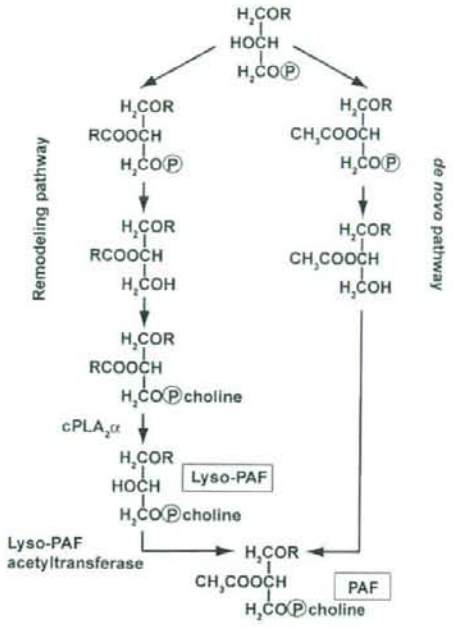


Fig. 2. Synthetic pathways of PAF. PAF is synthesized via two distinct pathways, the *de novo* and remodeling pathways. Lyso-PAF acetyltransferase catalyzes the final reaction for PAF synthesis in the remodeling pathway.

be explored here. Finally, we will highlight the lipid mediator PAF, which we have recently identified as playing a significant role during bone metabolism [40].

## 2. Role of prostanoids in bone metabolism and disease

### 2.1. $PGE_2$

#### 2.1.1. In vitro actions of $PGE_2$ on bone metabolism

*2.1.1.1. $PGE_2$ in osteoblasts: production and effects.* Within bone, $PGE_2$ is primarily produced by osteoblasts. More importantly, $PGE_2$ acts in an autocrine fashion on osteoblasts to form osteoclasts *in vitro*, leading to bone resorption [38]. Some of these *in vitro* experiments utilize the organ cultures of bones such as fetal/neonatal calvariae and limb bones [41–43]. The other experiments use the cocultures of primary osteoblasts and osteoclast precursor cells, which are derived from calvaria and either bone marrow or spleen, respectively [44,45].

A large number of cytokines, growth factors, and hormones are known to enhance $PGE_2$ production by affecting cPLA$_2\alpha$-, COX-2- and/or mPGES-1 in osteoblasts (Table 1). Many of these molecules such as IL-1 and IL-6 potentiate bone resorption [37], whereas other cytokines such as IL-4 [46,47] and IL-13 [47] have been reported to inhibit bone resorption by suppressing COX-2-dependent $PGE_2$ production in mouse osteoblasts. Throughout these studies, the role

Table 1
Molecules that enhance cPLA$_2\alpha$, COX-2, and/or mPGES-1-dependent $PGE_2$ production in osteoblasts

| Effector | Class | Affected enzyme(s) | Reference |
|---|---|---|---|
| IL-1α | Cytokine | COX-2 | [288,289] |
| | | COX-2 and mPGES-1 | [180] |
| IL-6 | Cytokine | COX-2 | [90] |
| TNF-α | Cytokine | cPLA$_2\alpha$ and COX-2 | [46] |
| PDGF | Growth factor | cPLA$_2\alpha$ | [289] |
| Basic FGF | Growth factor | COX-2 | [290] |
| BMP-2 | Growth factor | COX-2 | [291] |
| PTH | Hormone | COX-2 | [288] |
| 1α,25-(OH)$_2$ Vitamin D3 | Hormone | COX-2 | [45] |
| LPS | Pathogen | COX-2 and mPGES-1 | [180] |
| $PGE_2$ | Lipid | COX-2 | [288] |
| | | cPLA$_2\alpha$ and COX-2 | [100] |

of cPLA$_2\alpha$/COX-2/mPGES-1-mediated $PGE_2$ production by osteoblasts in bone resorption is clear.

In addition to bone resorption activity through the osteoblast-mediated osteoclastic differentiation, $PGE_2$ displays bone-forming activity in osteoblast monocultures. For example, $PGE_2$ stimulates the formation of mineralized bone nodules (lumps of extracellular mineralization *in vitro* that mimic calcification *in vivo*) and the activity of alkaline phosphatase (ALP; a differentiation maker of osteoblasts) in primary rat calvarial osteoblasts [48,49] and mouse osteoblastic MC3T3-E1 cells [50]. Increasing the extracellular calcium concentration in primary mouse osteoblasts can induce COX-2 expression and $PGE_2$ production through the protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) signaling pathways [51,52]. Consequently, the produced $PGE_2$ stimulates osteoblastic differentiation [52]. Bone cells sense interstitial fluid shear stress upon mechanical loading of bone through fluid flow [53], which is an important mechanism for the anabolic effect of mechanical loading. Runx2/Cbfa1, a transcriptional factor required for osteoblastic differentiation (see Section 2.1.1.4 for detail), induces COX-2 expression in response to fluid sheer stress in the cells [54]. This transcriptional factor is phosphorylated and activated by the ERK pathway [55,56]. Thus, it is consistent that the PKA and ERK signaling pathways in mouse osteoblastic MC3T3-E1 cells becomes activated by fluid shear stress, resulting in increases of COX-2 expression and $PGE_2$ release [57–59]. The produced $PGE_2$ accounts, at least in part, for the anabolic effect of mechanical loading [60,61].

*2.1.1.2. $PGE_2$ receptors in osteoblasts.* There have been four $PGE_2$ receptors identified (EP1–EP4) [13]. By evaluating the functional effects of EP subtype-specific ligands, the presence of EP1, EP2, and EP4 receptors have been shown in mouse osteoblastic MC3T3-E1 cells [62]. EP1 and EP4 transcripts have been detected in MC3T3-E1 cells by

Northern blot [62], while reverse transcriptase–polymerase chain reaction analysis revealed that mouse osteoblasts isolated from calvariae expressed transcripts of all four receptors with the rank order being EP4 > EP1 > EP2 > EP3 [63]. In human osteoblasts, only EP3 and EP4 were observed immunohistochemically [64].

Signaling through EP receptors activates two major intracellular pathways, the cyclic AMP (cAMP)-dependent pathway and the intracellular calcium-dependent pathway. The EP2 and EP4 receptors are known to mediate the stimulation of adenylate cyclase [65]. The binding of $PGE_2$ to EP2 and EP4 receptors induces COX-2 mRNA transcription in primary mouse osteoblasts through cAMP-dependent activation of PKA [66]. Stimulation of EP4 (and somewhat EP2) by specific agonists increases the activity of mouse calvarial osteoblasts and induces the transcription of receptor activator of NF-κB ligand (RANKL; see Section 2.1.1.3 for detail) and the subsequent osteoclast formation [43]. Analysis of the four EP receptor-deficient mice revealed that $PGE_2$ stimulates bone resorption in the cultured calvariae through the EP4-cAMP signaling pathway [63]. EP4 expression on mouse osteoblasts is required for osteoclast formation stimulated by lipopolysaccharide (LPS) and proinflammatory cytokines such as IL-1α, TNF-α and basic FGF in the coculture of primary mouse osteoblasts and bone marrow cells [67]. However, EP2 has also been reported to be indispensable in $PGE_2$-induced cAMP formation in primary mouse osteoblasts [66,68], which partially contributed to an increase in RANKL mRNA expression in cultured calvariae [68] and primary osteoblasts [69]. $PGE_2$-stimulated osteoclast formation in cultures of calvarial osteoblastic cells and spleen cells was reduced by about 90%, when osteoblasts were derived from EP2-deficient mice [70].

In addition to its role in osteoclast formation, EP4 has also been implicated in osteoblast differentiation. $PGE_2$ has been shown to stimulate differentiation of mouse calvarial osteoblasts by activating EP4 [71]. In mouse osteoblastic MC3T3-E1 cells, EP4 and possibly EP2 mediate differentiation of the cells [50,62].

EP1 mediates the intracellular calcium influx [65]. $PGE_2$ signaling through the EP1 receptor is important for many aspects of bone metabolism. Through this pathway, $PGE_2$ induces its own production, a process called $PGE_2$ autoamplification (see Section 2.1.1.3 for detail) in MC3T3-E1 cells [72]. Mineralized bone nodules also develop in response to EP1 signaling in primary rat osteoblasts [73], suggesting a role of EP1 in osteoblastic differentiation. These cells also increase their production of fibronectin, which is important during the early stages of bone formation [74].

We are unaware of any reports of EP3-regulated osteoblast function.

*2.1.1.3. Production of bone-resorbing factors by $PGE_2$ in osteoblasts.*

*2.1.1.3.1. RANKL.* Various systemic hormones (such as $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ and PTH), growth factors and cytokines increase the ability of osteoclasts to break down bone through osteoblasts [33,75]. Such externally regulated osteoclastic bone resorption is primarily dependent on the cell surface interaction between RANKL on osteoblasts and RANK on osteoclasts [76,77]. Osteoclast formation can be inhibited by osteoprotegerin (OPG), a decoy receptor that binds to RANKL and prevents its interaction with RANK [78]. $PGE_2$ can stimulate RANKL production [43,70] and inhibit OPG production [79,80] in osteoblasts in a cAMP-dependent manner.

*2.1.1.3.2. IL-6.* IL-6 has been reported to stimulate bone resorption [81,82]. $PGE_2$ induces the production of IL-6 in mouse and rat osteoblasts [83–85] through EP1 and EP2 signaling [83]. $PGE_2$ activates the IL-6 promoter through the cAMP-PKA dependent pathway [85]. Therefore, $PGE_2$ may enhance osteoclast formation through the production of IL-6 in osteoblasts.

IL-6 signaling is mediated by membrane bound IL-6 receptor (IL-6R) or soluble IL-6R (sIL-6R) that lacks transmembrane and cytoplasmic regions of IL-6R [86]. Because mouse primary osteoblasts express low levels of membrane bound IL-6R mRNA, IL-6 treatment alone cannot induce osteoclast formation in the mouse coculture of bone marrow cells and osteoblasts [87]. However, the coadministration of sIL-6R and IL-6 triggers osteoclast formation. In another report, simultaneous application of IL-6 and sIL-6R to mouse cultured calvariae enhanced bone resorption with the increased expression of RANKL mRNA and protein [88]. Since sIL-6R is present in human sera [89], it is possible that the sIL-6R/IL-6 complexes enable osteoblasts to promote osteoclast formation *in vivo*.

Contrary to the $PGE_2$-stimulated IL-6 production, IL-6 can also increase COX-2 expression and $PGE_2$ production in MC3T3-E1 cells [90]. Thus, there appears to exist a synergistic interaction between IL-6 and $PGE_2$ during osteoclast formation *in vivo*.

*2.1.1.3.3. IL-1.* The two forms of IL-1, IL-1α and IL-1β, are also potent bone-resorbing cytokines [91,92]. IL-1β mediates $PGE_2$-stimulated mouse osteoclast formation by osteoblasts [93]. Indeed, $PGE_2$ induces IL-1β gene expression and protein production in mouse osteoblasts through the cAMP-PKA pathway [93,94]. Conversely, IL-1β can also stimulate $PGE_2$ production in mouse primary osteoblasts cocultured with bone marrow cells [80]. This is consistent with other reports that IL-1 induces osteoclast formation by a mechanism involving $PGE_2$ in mice and rats [95,96]. Suppression of the OPG production in osteoblasts by the autocrine $PGE_2$ is one of the critical mechanisms of IL-1β-induced osteoclast formation [80,96]. Taken together, an osteoblastic positive feedback loop composed of $PGE_2$ and IL-1 appears to regulate osteoclast formation and bone resorption through a RANKL-dependent mechanism. This synergism for osteoclast formation is similar to that between $PGE_2$ and IL-6. However, Jimi et al. reported a RANKL-independent mechanism by which IL-1 directly affects the differentiation and function of osteoclasts [97,98].

*2.1.1.3.4. $PGE_2$.* $PGE_2$ can amplify its own production in mouse osteoblastic MC3T3-E1 cells [72,99,100]. This phenomenon, termed "autoamplification", is accompanied by an increase in $cPLA_2\alpha$ and COX-2 protein levels. The activation of EP2 and EP4 receptors results in COX-2 mRNA transcription in primary mouse osteoblasts [66]. Another study using specific EP agonists demonstrated that the EP1 receptor was responsible for $PGE_2$ autoamplification in MC3T3-E1 cells [72]. This autoamplification system is thought to be essential for maintaining $PGE_2$ production and prolonging the effects of short-lived $PGE_2$ during bone-resorptive disorders, such as long-term immobilization and bone inflammation.

*2.1.1.3.5. Proteinase.* It has been proposed that osteoblast-secreted proteases can control the access of osteoclasts to the bone surface [101]. Mouse osteoblastic MC3T3-E1 cells secrete collagenase to degrade collagen [102], a crucial step in initiating bone remodeling [103]. $PGE_2$-stimulated bone resorption is accompanied by the induction of two matrix metalloproteinases (MMPs), MMP-2 and MMP-13, in mouse calvarial cultures [63]. Furthermore, Kim et al. reported that $PGE_2$ enhanced the mRNA expression of MMP-1, an interstitial collagenase, in mouse primary osteoblasts [104]. One group of metalloproteinases, a disintegrin and metalloproteinase (ADAM), has both metalloproteinase (proteolytic) and disintegrin (adhesion) domains [105]. $PGE_2$ has been shown to stimulate the expression of a new member of the ADAM family, a disintegrin and metalloproteinase with thrombospondin motif-1 (ADAMTS-1) in osteoblast-enriched femoral metaphyseal region of male rats injected with PTH [106].

*2.1.1.4. Production of bone-forming factors by $PGE_2$ in osteoblasts*

*2.1.1.4.1. Runx2/Cbfa1.* Runx2/Cbfa1 is a transcription factor essential for osteoblastic differentiation [107]. $PGE_2$ induces the expression of Runx2/Cbfa1 through EP4 receptor activation in mouse osteoblasts, resulting in the enhanced formation of mineralized nodules [108].

*2.1.1.4.2. Bone morphogenetic protein.* The bone-forming effects of $PGE_2$ are likely mediated in part by other molecules. Bone morphogenetic proteins (BMPs) are crucial in skeletal development and repair [109]. They stimulate mouse osteoblast formation from mesenchymal progenitors and osteoblastic differentiation by increasing Runx2/Cbfa1 expression [110]. $PGE_2$ induces BMP-2 mRNA expression by binding to the EP4 receptor in human mesenchymal stem cells that are capable of differentiating into osteoblasts [111].

*2.1.1.4.3. Extracellular matrix.* Adhesive interactions between osteoblasts and extracellular matrix components, including type I collagen, fibronectin and bone sialoprotein, are important for osteoblast survival, proliferation, and differentiation [112]. $PGE_2$ enhances collagen synthesis in mouse osteoblasts [113]. Fibronectin is a heterodimeric bone-matrix glycoprotein that promotes the survival of differentiated osteoblasts [114]. Like collagen, fibronectin production is stimulated by $PGE_2$ in rat osteoblasts [74]. Bone sialoprotein is a highly sulfated, phosphorylated, and glycosylated protein that can bind to hydroxyapatite and mediate cell attachment through an RGD sequence. Bone sialoprotein has a potent role in the initiation of bone mineralization. $PGE_2$ also stimulates the bone sialoprotein mRNA transcription in rat osteoblasts [115].

*2.1.1.5. Effects of $PGE_2$ on osteoclasts.* In addition to the indirect effects of $PGE_2$ on osteoclastic differentiation through osteoblasts, $PGE_2$ exerts direct effects on both immature osteoclast precursor cells and mature osteoclasts.

*2.1.1.5.1. Osteoclast differentiation.* $PGE_2$ enhances the differentiation of mouse bone marrow-derived macrophages into osteoclasts synergistically with RANKL [116]. Kobayashi et al. reported that mouse bone marrow-derived macrophages express EP1, EP2, and EP4 [117]. Osteoclastic differentiation of RAW 264.7 cells was also induced by RANKL treatment and $PGE_2$ stimulated the differentiation even further through the EP2/EP4 receptors [118]. $PGE_2$ also enhanced macrophage colony-stimulating factor (M-CSF)/RANKL-induced osteoclast formation in mouse macrophage cultures [119]. In contrast to mouse osteoclast formation, the direct effect of $PGE_2$ on human osteoclasts is controversial. Lader et al. reported that $PGE_2$ stimulates osteoclast formation in human bone marrow cell cultures treated with M-CSF, TNF-$\alpha$ and IL-1 [120], whereas Chenu et al. showed that $PGE_2$ inhibits osteoclast formation in human bone marrow cell cultures treated with $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ [121]. Because of the lack of receptors for $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ in osteoclasts [122], this hormone is not expected to have direct effects on these cells. In the report of Chenu et al., therefore, osteoblast-like cells in the bone marrow cultures may mediate the effects of $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ on osteoclasts. Meanwhile, M-CSF, TNF-$\alpha$ and IL-1 could stimulate osteoclasts directly under the similar experimental conditions of Lader et al. [120,123]. Thus, it is possible that the different extracellular stimuli resulted in different expression profiles of EP receptor subtypes in osteoclasts. This may account for the apparent discrepancy regarding the effect of $PGE_2$ between these two reports.

In addition to bone marrow cell cultures, $PGE_2$ has been demonstrated to inhibit RANKL/M-CSF-induced osteoclast formation in human peripheral blood mononuclear/$CD14^+$ cell cultures [119,124]. This suggests that $PGE_2$ may stimulate the production of an unknown inhibitory factor(s) for osteoclast formation in human $CD14^+$ cells. This inhibitor production appeared to involve the EP2/EP4-cAMP-PKA signaling pathway [119].

The EP2 and EP4 receptors are down-regulated during the differentiation of mouse bone marrow-derived macrophages into osteoclasts [117]. Treatment of EP4-transfected osteoclasts with $PGE_2$ inhibited the formation of both actin rings (an actin-rich large ring-like structure around the periphery of osteoclasts) and resorption pits (dentin holes formed by osteoclasts). This suggests that the loss

of EP2/EP4 signaling during osteoclast formation enabled mature osteoclasts to escape the inhibitory effects of $PGE_2$ on bone resorption. It is notable that calcitonin, a bone resorption-inhibiting hormone, impairs the formation of actin rings and resorption pits in a cAMP-PKA dependent manner [125]. The function of EP1 in mature osteoclasts remains unknown.

*2.1.1.5.2. Mature osteoclasts.* $PGE_2$ inhibits bone resorption by isolated mature rabbit osteoclasts by activating adenylate cyclase through the EP4 receptor [126]. Both EP3 and EP4 receptors have been detected immunologically in mature human osteoclasts [64,127]. As described above, the expression of EP4 receptor is suppressed in mouse osteoclasts formed *in vitro* so that the cells escape the inhibitory effects of $PGE_2$ on bone resorption [117]. Therefore, it is possible that authentic osteoclasts express EP4 and decrease their function in response to $PGE_2$. Indeed, the activation of EP4 inhibits actin ring formation in human mature osteoclasts [127]. Meanwhile, EP3 stimulation increases the number of lamellipodium-harboring osteoclasts [127]. Because lamellipodia are structures responsible for cell movement, EP3 may have a role in the motility of mature osteoclasts.

*2.1.2. In vivo actions of $PGE_2$*

Investigation of the four EP receptor knockout mice has elucidated the actions of $PGE_2$ under various physiological and pathological conditions [65]. In addition, highly selective agonists and antagonists for the $PGE_2$ receptors have been developed [65]. The use of these experimental tools is paramount for understanding the complicated function of $PGE_2$ during bone metabolism and disease.

*2.1.2.1. Bone abnormalities in EP-deficient mice under physiological conditions.* Although the skeletons of EP4-deficient mice (at 4–5 months of age) are normal, an imbalance in bone remodeling is observed in male mice at 15–16 months of age [128]. These mice suffer from a deficiency in bone formation due to a defect in osteoblast formation. EP4-deficient mice also exhibit reduced structural strength and trabecular bone volume, despite having normal body weight and bone size [129]. EP2-deficient mice have abnormally weak bones, whereas EP1 receptor knockouts appear normal [130].

*2.1.2.2. Pharmacological effects of $PGE_2$ on bone metabolism.* As mentioned earlier (Section 2.1.1), $PGE_2$ plays an important role in bone metabolism *in vitro*. However, the number of *in vivo* studies that focus on the pharmacological effects of $PGE_2$ on bone metabolism is few. The anabolic properties of $PGE_2$ have been analyzed by systemically administrating $PGE_2$ to rats [131,132]. Another study demonstrated that exogenously administered $PGE_2$ increases bone formation in response to mechanical loading of the tibia [61]. However, bones from EP4-deficient mice were unresponsive to exogenously administrated $PGE_2$ [108]. Furthermore, the EP4 agonist ONO-4819 prevented bone loss and restored bone mass and strength in rats subjected to ovariectomy and immobilization [108]. The EP2 receptor selective agonist CP-533,536 also stimulated local bone formation at trabecular, endocortical, and periosteal surfaces in rats [133]. In contrast to this anabolic effect, EP2 was also shown to mediate $PGE_2$-stimulated hypercalcemia in a study of EP2-deficient mice [68].

*2.1.2.3. Role of $PGE_2$ in bone disease.* Consistent with the anabolic effects of exogenous $PGE_2$, endogenous $PGE_2$ may participate in the recovery from osteoporosis and bone fractures. However, endogenous $PGE_2$ has also been implicated in several bone-resorptive inflammatory disorders.

*2.1.2.3.1. Bone fracture.* Fracture healing is a complicated process that includes the proliferation and differentiation of mesenchymal stem cells into chondrocytes and osteoblasts. Healing is complete when mature lamellar bone is formed after woven-bone bridges the bone gap [134]. Endogenous levels of $PGE_2$ and COX-2 are increased locally after fracture in experimental animals [135,136]. Several studies have shown that the COX inhibitors, *i.e.*, nonsteroidal anti-inflammatory drugs (NSAIDs), delay bone repair [137,138]. In addition, fracture healing is delayed in COX-2-deficient mice compared with COX-1-deficient and wild-type mice [139]. In this study, osteoblast formation was impaired and Runx2/Cbfa1 expression was reduced in bone marrow stromal cell cultures from COX-2-deficient mice [139]. The addition of $PGE_2$ rescued the defects observed in COX-2-deficient cells. $PGE_2$ improves fracture healing through EP4 receptor signaling in mice [128]. In the absence of EP4 receptor, aged mice suffer from decreased bone mass and an impaired ability to heal from fractures. The EP2 receptor selective agonist CP-533,536 is able to improve the healing process in rats and dogs, suggesting that the EP2 receptor contributes to the anabolic activity of bone in response to $PGE_2$ as well [133,140].

An optimal mechanical stress at the fracture site is essential to achieve prompt and complete healing of a fracture [141]. $PGE_2$ and COX-2 promote bone formation in response to mechanical loading [60,61]. Taken together, $PGE_2$ has bone-forming activity during bone repair.

*2.1.2.3.2. Osteoporosis.* Osteoporosis is a skeletal disorder characterized by weakened bone strength, which increases the risk for fracture. The equilibrium between bone resorption and formation is maintained in young, healthy women. However, in patients suffering from postmenopausal osteoporosis, this equilibrium is shifted towards resorption due to an acute decrease in serum estrogen level after cessation of ovarian function [142].

Administration of $PGE_2$ can suppress bone loss in rats that have undergone ovariectomy or orchidectomy [143,144]. Following EP4 receptor activation, bone formation is stimulated and bone loss is prevented in ovariectomized rats [108]. Consistent with EP4-mediated osteoblastic differentiation of bone marrow cells *in vitro* [145] (see Section 2.1.1.2), the density of osteoblasts lining