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Topical application of porcine placenta extract inhibits the progression of experimental contact hypersensitivity

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ABSTRACT

Aim of study: Placenta extract features as a composition of ointments used for skin beautification, dermatological diseases and skin dryness. However, little evidence has been cited about its underlying mechanisms of action by which it exerts a beneficial role in dermatological diseases *in vivo*. In this study, we intended to test the effect of topical application of porcine placenta extract in mouse model of contact hypersensitivity and elucidate its mechanism of action.

Materials and methods: To test the *in vitro* effect of porcine placenta extract, RAW 264.7 cells were cocultured with porcine placenta extract and stimulated with LPS (1 μg/ml) and the expression of inflammatory mediator TNF-α was estimated by RT-PCR at the mRNA level and by intracellular staining at the protein level. To further test *in vivo* efficacy, porcine placenta extract was topically applied to the mice with experimental skin hypersensitivity. For *in vivo* studies placenta extract in gel form was topically applied to ear of DNCB-induced contact hypersensitivity mouse model everyday for 2 weeks and progression of the disease was estimated by following criteria: (a) ear thickness, (b) serum IgE level by ELISA, (c) histological examination of ear tissue by H&E staining and (d) cytokine profile of total cells and $CD4^+$ T cells by real time PCR.

Results: Topical application of porcine placenta extract on mouse ears with contact hypersensitivity decreased the severity and progression of the disease manifested by reducing ear swelling, inflammation and edema. Histological evaluation showed that placenta extract treatment reduced lymphocyte infiltration in the ear tissues. Protective effect of placenta extract is also associated with down-regulation of serum IgE level and inflammatory cytokine production (IL-1β, IFN-γ, TNF-α, IL-4, IL-12 and IL-17) in total lymph node cells and $CD4^+$ T cells.

Conclusions: Our data indicate that protective effect of porcine placenta extract in contact hypersensitivity is mediated by inhibition of the inflammatory responses and IgE production, suggesting a potential therapeutic application of porcine placenta extract to modulate skin inflammation.

1. Introduction

Placenta is a temporary organ present in females during gestation and supplies oxygen and nutrients to the developing fetus. The placenta is considered as a reservoir of cytokines, hormones, bioactive peptides, enzymes, growth factors, vitamins and minerals. The placenta is discharged from the mother's body when the fetus is born and the nutritional substances and vitamins can be extracted and is known as "placenta extract". Placenta extract (PE) has been used for wound healing in Chinese folk medicine. The medical uses and dermatological benefits of placenta extracts have been reported. Recently, benefits of the topical use of PE on chronic and non-healing wounds have been reported (Tiwary et al., 2006). Intramuscular injection of human PE commercially known as "Placentrex" also healed radiation mucositis developed in squamous cell carcinoma patients during radiation therapy (Kaushal et al., 2001). In addition, the anti-inflammatory effect of human PE has been observed in rheumatoid arthritis and carrageenin-induced

Abbreviations: PE, placenta extract; LPS, lipopolysachharide; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IFN-γ, interferon gamma; IL-4, interleukin 4; IL-6, interleukin 6; IL-2, interleukin 2; IL-17, interleukin 17; CHS, contact hypersensitivity; LC, Langerhan's cell; APC, antigen presenting cell; DLN, draining lymph node; NK cells, natural killer cells; DNCB, 2,4-dinitrocholorobenzene; OX, oxazolone; PBS, phosphate buffer saline; LPS, lipopolysachharide; PMA, phorbol 12-myristate 13-acetate.

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edema (Banerjee et al., 1992; Rosenthal, 1982). The mechanism of its anti-inflammatory action has been shown to exert in part by inhibiting platelet aggregation (Kumar et al., 2003). Human PE also features as a component of various skin ointments and used for skin vitalizing, nourishment, melanocyte growth and pigment inducing activities (Pal et al., 2002) as well as for the treatment of skin hypersensitivity like dermatitis and psoriasis. However, components and medicinal efficacy are not clarified in detail. In addition, the molecular mechanism of the immunomodulatory effects of PE in healing skin inflammation and hypersensitivity reactions is still not clearly known. Interestingly it is known that during pregnancy there is a shift of immune response to $CD4^+$ T helper type 2 (Th2) cells (Dudley et al., 1993; Sacks et al., 2001) that help survival of fetus (Elenkov et al., 2001; Krishnan et al., 1996; Marzi et al., 1996). The immunomodulatory effect of pregnancy hormone estradiol has also been described in sclerosis patients (Soldan et al., 2003). These findings suggest that the immunomodulatory effect of PE is probably manifested in circumventing an inflammatory Th1 type immune response. Although, the effect of human PE is largely studied, only few scientific reports investigated the effect of PE from other animals like pig, sheep and horses. Placenta extracts from animal are also available commercially and used regularly in improving skin functions in the form of various ointments and lotions. For example, the wound healing effect of porcine PE on rats with thermal injury has been reported (Wu et al., 2003). However, the effect of porcine PE on skin inflammation mediated by immune cells has not been investigated. Taken together, we chose to investigate the effect of porcine PE in preventing or reducing a Th1 type skin inflammation, namely contact hypersensitivity (CHS).

Contact hypersensitivity (CHS) is an epidermal T cell-mediated inflammatory response to low molecular weight haptens. CHS is executed by the recognition and capture of haptens by Langerhan's cells (a type of dendritic cell which functions as skin antigen presenting cell (APC)). Langerhan's cells present captured antigens to naïve T cells, which leads to activation and clonal proliferation of inflammatory Th1 type cytokines (Watanabe et al., 2002), which mediates inflammatory immune disorders. In this study, we investigated the effect of topical application of aqueous fraction of porcine PE on experimental CHS responses and elucidated the mechanism of action. Topical application of porcine PE suppressed disease progression by down-regulation of serum level IgE and inflammatory cytokine production.

2. Materials and methods

2.1. Pig placenta and preparation of placenta extract

Pig placentas were obtained immediately after vaginal deliveries, at term, from uncomplicated pregnancies; amnion and cord were discarded and the remaining tissue was exhaustively washed in cold 0.9% NaCl to remove all traces of blood. The isolation and extraction procedures were carried out at 4 °C. Preliminary homogenates (50%, w/v) were prepared in phosphate-buffered saline (PBS, 0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, and 0.14 M NaCl, pH 7.4) with the use of a knife homogenizer (Daesung Artron, Korea) and a Polytron Homogenizer (ART-moderne Labortechnik, Germany). To prepare soluble fraction, tissue homogenates were centrifuged at 5000 rpm for 15 min and protein concentration was determined in the supernatants, with the use of BCA Protein Assay Reagent Kit (Pierce, USA). The soluble aqueous fractions were used for further experiments as "homogenates" and refrigerated as 1 ml aliquots. The relative percentage of amino acids in the aqueous fraction of porcine placenta extract are: Asp (0.06%), Thr (0.03%), Ser (0.03%), Glu (0.09%), Pro (0.03%), Gly (0.03%), Ala (0.04%), Val (0.04%), Ile (0.03%), Leu (0.06%), Tyr (0.03%), Phe (0.03%), His (0.03%), Lys (0.05%).

Table 1
Composition of vehicle and placenta extract gel.

Components	Compostion of gels (1 g)	
	Vacant gel	Placenta gel
Placenta Extract	0 mg	100 mg
Carbopol 934P	10 mg	10 mg
Poloxamer 407	30 mg	30 mg
Propylene glycol	150 μl	150 μl
Distilled water	Adequate	Adequate
Ethanol	50 μl	50 μl
L-Methanol	30 μl	30 μl

2.2. Preparation of placenta extract gel for topical application in CHS

Carbopol 934P (1%, w/v) (Samkwang Chemical, Chinhae, Kyongnam, Korea), Poloxamer 407(3%, w/v) and propylene glycol (15%, w/v), L-menthol (3%, w/v) (Sigma Chemical Co., St Louis, MO, USA) and ethanol (5%, w/v) were used for making the gel base of porcine placenta extract (Oh et al., 2006). Placenta extract gel was prepared by adding 100 mg of the extract to 1 ml of the gel base. The composition is summarized in Table 1.

2.3. Cell culture

The mouse macrophage cell line, RAW 264.7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, USA), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) in a humidified 37 °C incubator. Cells were co-cultured with placenta extract for the indicated period of time and stimulated with 1 μg/ml LPS (Sigma) for 8 h for all analysis.

2.4. Cytotoxicity assay

Trypan blue exclusion method was employed to perform cytotoxicity assay by determining number of viable cells microscopically. Briefly, Raw 264.7 cells were cocultured with 10% placenta extract and after 0, 8, 12 and 24 h 100 μl of cell suspension was stained with Trypan Blue. Percentage of viable cells was determined by an equation: 100 − [(No. of cells including the stain × 100)/total no. of cells]. Untreated cells were used as controls and calculations were done in triplicate.

2.5. Intracellular cytokine staining

Intracellular levels of TNF-α was analyzed by flow cytometry as described previously (Suni et al., 1998). Briefly, RAW 264.7 cells (2×10^6 cells/well) were stimulated with LPS (1 μg/ml) for 8 h and 1 μg/ml brefeldin A (Sigma) was added 10 h before the end of culture. Cells were incubated with phycoerythrin (PE)-labeled anti-TNF-α (BD Pharmingen, USA; 559503) antibody for intracellular molecular detection. Control histograms were made by staining with the relevant isotype control antibody. After staining in the dark at 4 °C for 30 min, cells were washed and resuspended in PBS, and at least 10,000 events were analyzed using EPICS XL™ and EXPO32™ software (Beckman Coulter, CA, USA).

2.6. Animals and experimental design

Female BALB/cJ mice (6- to 8-week old) were brought from Orient Bio (Korea) and maintained under specific pathogen-free

conditions in the animal facility at Gwangju Institute of Science and Technology (GIST). The animal experiments were approved by the GIST Animal Care and Use Committee.

2.6.1. Induction of experimental contact hypersensitivity in mouse ear

For the induction of contact hypersensitivity (CHS), mice were divided into 3 groups with 5 mice per each treatment group; normal healthy control treated with PBS alone without CHS induction, treatment groups treated either with vacant gel or gel containing placenta extract. CHS was induced by painting 20 μl (per ear lobe) of 1% 2,4-dinitrocholorobenzene (DNCB) after stripping each ear lobe with surgical tape. Control group mice were painted with PBS after ear stripping. DNCB painting was done after every 2 days in a week for 1 month. At the end of 2 weeks, serum IgE concentration was estimated to check induction and thereafter treatment was done every day by painting 50 μl (per ear lobe) of vehicle (vacant gel) or gel containing placenta extract (PE group). Ear thickness was measured 24 h after each application of DNCB with calipers. To confirm the inefficacy and inertness of vehicle (vacant gel), vehicle and PBS were applied separately to DNCB-induced CHS mice. Both groups failed to enhance dermatopathological symptoms of CHS.

2.7. Histological observation

Ears excised from mouse of each group were fixed with 4% paraformaldehyde for 16 h and embedded in paraffin. Paraffin embedded ears were sectioned into 6 μm thin sections and stained with hematoxylin (Sigma) and eosin (Sigma) (H&E). Infiltrated lymphocytes, thickening of the epidermis were observed under microscope and photographed.

2.8. Measurement of serum IgE

Blood was obtained from tail vein of mouse at 2 weeks (to check disease induction) and at the end of 4 weeks (to check effect of PE treatment on disease progression). Total serum IgE was measured by ELISA kit (BD Biosciences) with serum diluted 200-fold. Detection antibody and streptavidin conjugated HRP were diluted 500- or 250-fold, respectively and absorbance was measured at 490 nm. Serum total IgE concentration of each sample was determined from a standard curve of mouse IgE.

2.9. Isolation of total draining lymph node cells and $CD4^+$ T cell

Draining lymph nodes including superficial, cervical, axillary and brachial lymph nodes were isolated from each group of mouse and single cell suspensions were prepared by mechanical dispersion according to standard procedures (Kirk et al., 2001). A part of the cells was kept separately as total draining lymph node cells and another part was used to isolate $CD4^+$ T cells with $CD4^+$ T cells isolation bead and column (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's protocol. Both types of cells (total and $CD4^+$ draining lymph node T cells) were maintained (5×10^6 cells/well) in primary T cell media containing DMEM (Invitrogen) supplemented with 10% FBS (Hyclone), 3 mM L-glutamine (Sigma), 10 mM HEPES (Sigma), 100 U/ml streptomycin and penicillin (Sigma) and 0.05 mM 2-beta-mercaptoethanol (Sigma). Cells were stimulated with PMA (20 nM) and ionomycin (2 μM) for 2 h.

2.10. RNA isolation and RT-PCR analysis

Total RNA was isolated from PMA and ionomycin stimulated $CD4^+$ T cells and total draining lymph node cells or LPS stimulated RAW 264.7 cells by TRIZol (Molecular Research Center) according to the manufacturer's protocol. cDNA was prepared using the Improm-II reverse transcription system (Promega, Madison, WI, USA). cDNA was subjected to quantitative real-time PCR using SYBR Premix Ex Taq (Takara, Shiga, Japan) with primers described in Table 2 in DNA Engine with a Chromo-4 Detector (MJ Research, Waltham, MA, USA). The data were normalized using the expression levels of HPRT. Relative expression level of the gene in the experimental group was compared with that of the control group. Results are expressed as percentage expression of the genes which was calculated taking the expression of the gene in vehicle group as 100%.

Table 2
Primer sequences used for real-time PCR.

Genes	Sequences (5′–3′)	T_m (°C)
HPRT	F: TTATGGACAGGACTGAAAGAC	53.9
	R: GCTTTAATGTAATCCAGCAGGT	54.4
IL-2	F: CCTGAGCAGGATGGAGAATTACA	58.4
	R: TCCAGAACATGCCGCAGAG	58.4
IL-1β	F: TTGAAGAAGAGCCCGTCC	54.4
	R: CTTATGTTCTGTCCATTGAGGT	53.3
TNF-α	F: CATCTTCTCAAAATTCGAGTGACAA	58.4
	R: TGGGAGTAGACAAGGTACAACCC	59.2
IFN-γ	F: TCAAGTGGCATAGATGTGGAAGAA	56.6
	R: TGGCTCTGCAGGATTTTCATG	53.3
IL-4	F: ACAGGAGAAGGGACGCCAT	55.4
	R: GAAGCCCTACAGACGAGCTCA	55.4
IL-12p40	F: GGAAGCACGGCAGCAGAATA	57.5
	R: AACTTGAGGGAGAAGTAGGAATGG	58.7
IL-17A	F: TTCATCTGTGTCTCTGATGCT	53.9
	R: TTGACCTTCACATTCTGGAG	53.3

2.11. Statistical analysis

A two-tailed Student's *t*-test was employed where $p < 0.05$ was considered to be statistically significant (*<0.05, **<0.005, ***<0.001).

3. Results

3.1. Porcine placenta extract inhibits macrophage activation

We initially performed a cytotoxicity test to define the optimal concentration at which placenta extract (PE) did not induce cell damage. PE treatment up to 10% did not induce cell death in macrophage cell line RAW 264.7 cells. Cells were incubated with 10% of PE and cell viability was measured at 0, 8, 12 and 24 h by trypan blue exclusion method. Untreated cells were taken as control (Fig. 1A). LPS stimulation induces RAW 264.7 cell activation with a change in morphology, a signature of macrophage activation during inflammatory responses (Kim et al., 2007). We tested whether treatment of PE could inhibit macrophage activation, thereby reducing the production of inflammatory molecules. RAW 264.7 macrophage cells were pretreated with PE for overnight and stimulated with LPS (1 μg/ml) for additional 8 h. The effect of PE treatment on changes in cell morphology (as an activation marker) and in the expression level of TNF-α, proinflammatory cytokine, was measured. PE treatment significantly reduced macrophage activation upon LPS stimulation monitored by changes in cell morphology (Fig. 1B). This indicates that PE can retain the wild type morphology of RAW 264.7 cells presumably by inhibiting their activation. Since activated macrophage cells upon LPS stimulation produce high levels of TNF-α, we also tested whether PE treatment could reduce TNF-α production upon LPS stimulation. Indeed, PE treatment significantly decreased TNF-α mRNA (Fig. 1C) and protein (Fig. 1D) levels compared to the control (without PE treatment, W/O) cells. Together with IL-1β, TNF-α plays a pivotal role

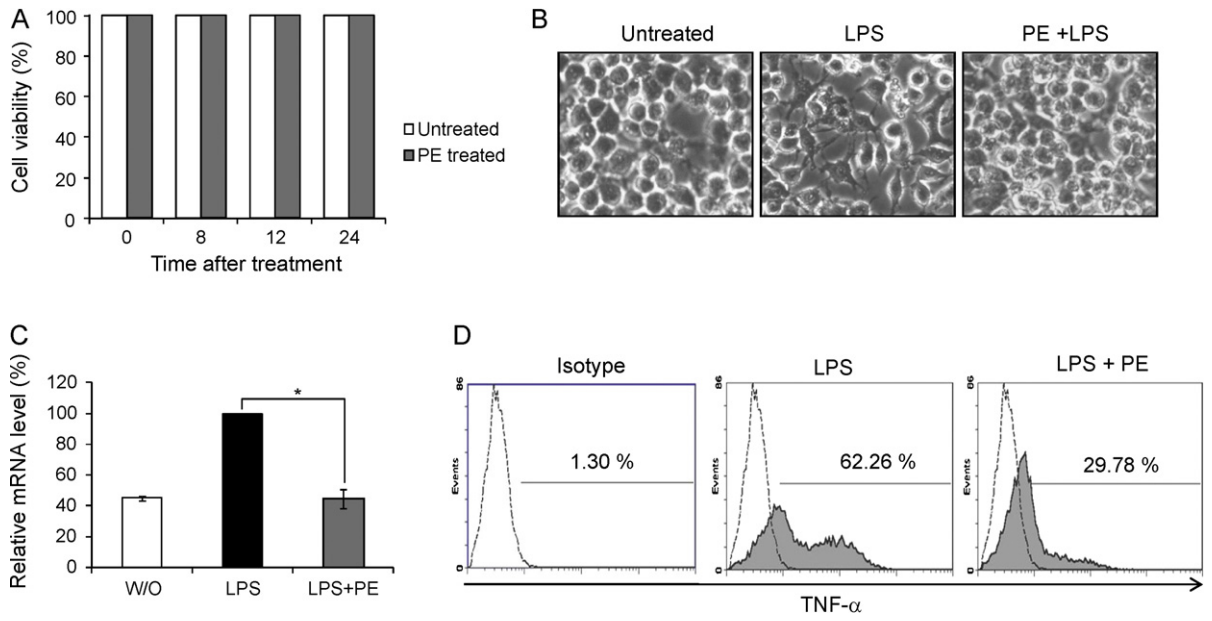

Fig. 1. Effect of porcine placenta extract on the activation of macrophage cells. (A) To test the cytotoxicity of porcine PE in mouse macrophage cell line, RAW 264.7 cells were cocultured with 10% PE for 8, 12 and 24 h. Cell viability was estimated at the indicated time points by Trypan Blue exclusion method. At each point untreated cells were used as control. (B) Porcine PE inhibits activation-dependent morphological change of RAW 264.7 cells upon LPS stimulation. Cells were cocultured overnight with 10% PE and then stimulated with LPS (1 μg/ml). Cells were visualized under microscope to monitor morphological changes. (C) RAW 264.7 cells were cocultured overnight with 10% PE and then stimulated with LPS (1 μg/ml). Total RNA was isolated and analyzed by quantitative real-time PCR. Relative expression level of the TNF-α in the LPS and PE + LPS samples were compared with the control group. Results are expressed as percentage expression of the genes by taking the expression of the gene in LPS treated sample as 100%. (D) RAW 264.7 cells (2×10^6 cells/well) were stimulated with LPS (1 μg/ml) for 8 h and 1 μg/ml brefeldin A (Sigma) was added 10 h before the end of culture. Intracellular TNF-α was analyzed by FACS. Error bars indicates SD. One (*) asterisk indicates $p < 0.05$. Data are representative of three independent experiments.

in skin inflammation and hypersensitivity response (Wang et al., 2001, 2003). Cumulatively, these data indicate that PE may have an anti-inflammatory effect through inhibition of macrophage activation.

3.2. Topical application of porcine placental extract inhibits the progression of experimental contact hypersensitivity

Since treatment of PE in RAW 264.7 macrophage cell line inhibited its activation and production of TNF-α we further investigated whether PE treatment could reduce the symptoms of contact hypersensitivity (CHS) in *in vivo* animal model. To apply PE in animal model of CHS, PE-containing ointment was prepared as described in Section 2. CHS was induced by DNCB treatment for 2 weeks and then treatment was initiated as indicated in Fig. 2. Mice were treated daily either with vehicle alone (vacant gel) or PE-containing gels till the end of experiment (see Section 2 in detail). The effect of PE treatment on CHS progression was compared with the control (vehicle) group. During the treatment period ear thickness was measured as a marker for clinical manifestation of CHS severity. Protective effect of PE treatment against CHS progression began to appear after 1 week (day 21) of PE gel treatment (Fig. 2). Mice treated with PE showed considerably decreased ear thickness and showed no further thickening of the ear compared to the vehicle group that showed continuous increase in ear thickness till the end of experiment (Fig. 2). The clinical symptoms of CHS include redness, edema, inflammation and dryness. Compared to control group, PE treated group showed significantly reduced symptoms of CHS. Chronic inflammation leads to an increase of local lymph node size. To check if the protective effect of PE treatment also affected immune response in local lymph nodes, draining lymph nodes were isolated from each treatment group and their size was compared between the treatment groups. PE group showed much smaller size of draining lymph nodes compared to vehicle group (Fig. 3). Since an increase in serum IgE levels is associated with clinical symptoms of CHS, we tested whether amelioration of CHS by PE treatment is also associated with modulation of serum IgE levels. Indeed, PE group showed significantly lowered serum IgE levels compared to control group (Fig. 4). The healing effect of topical application of PE in CHS was further confirmed by histological examination of the inflamed ear tissue by H&E staining. Compared to the PE group, vehicle group showed intensified inflammation manifested by thickening of the epidermis, fibrosis in the dermis and excessive invasion of inflammatory molecule in the ear tissue (Fig. 5). Collectively these data indicate a beneficial effect of placenta extract in modulation of contact hypersensitivity.

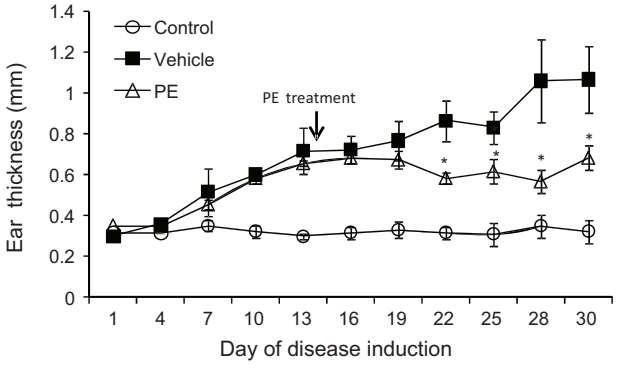

Fig. 2. Effect of porcine placenta extract on the ear thickness of mice induced to experimental contact hypersensitivity. CHS was induced by painting 20 μl (per ear lobe) of 1% 2, 4-dinitrocholorobenzene (DNCB) or PBS (for control group) every two days in a week for 1 month and at the end of 2 weeks, 50 μl (per ear lobe) of porcine placenta extract/vacant gel was topically applied every day for next 2 weeks (arrow indicates initiation of PE treatment) and progression of CHS was assessed by measuring ear thickness 24 h after each application of DNCB and plotted. Error bars indicates SD. One (*) asterisk indicates $p < 0.05$. Data are representative of three independent experiments.

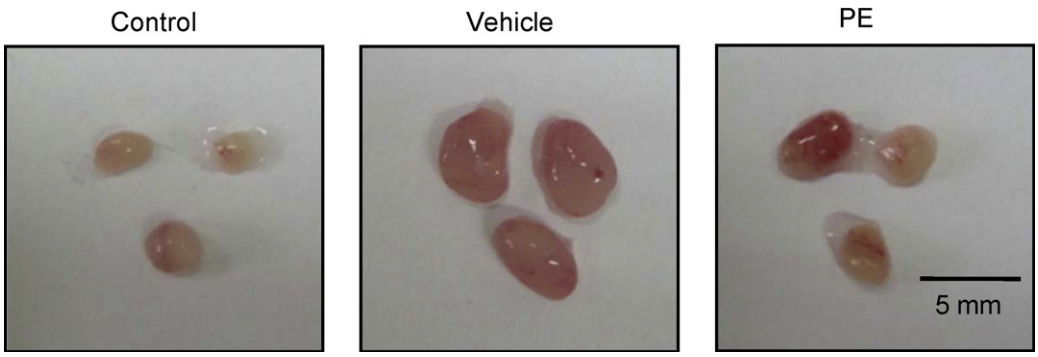

Fig. 3. Effect of porcine placenta extract on the size of ear draining lymph nodes. Prior to isolation of cells draining lymph nodes (DLNs) of representative groups of mice were photographed to show the effect of PE on DLN size.

3.3. Treatment of porcine placental extract down-regulates the expression levels of pro-inflammatory cytokines

CHS is mainly described as a Th1 type hypersensitivity response although other cell types are also involved. To test whether the protective effect of PE against CHS progression is mediated by changes in effector molecules of immune cells, we measured the cytokine profile from the total draining lymph node cells (Fig. 6A) and $CD4^+$ T cells (Fig. 6B). In accordance with results of reduced inflammation and edema in histological examination (Fig. 5), PE group showed significantly reduced expression levels of inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, IL-12 and IL-17 in total lymph node cells (Fig. 6A) and $CD4^+$ T cells (Fig. 6B). These results suggest that healing effect of PE treatment in CHS progression is mediated by down-regulation of pro-inflammatory cytokine and IL-4 levels in total lymph node cells as well as $CD4^+$ T cells.

4. Discussion

Our study demonstrates the potent immunomodulatory efficacy of porcine placenta extract (PE) in experimental contact hypersensitivity (CHS). Porcine PE treatment healed CHS symptoms such as skin edema, inflammation and lymphocyte infiltration by down-regulating the levels of pro-inflammatory cytokines as well as serum IgE.

Does PE have beneficial role in CHS? If yes, then what could be its potential mechanism of action? To answer the questions we started *in vitro* cell culture studies by testing whether PE can modulate the effector function of macrophage, the master regulator of innate immune response. Stimulation of mouse macrophage cell line (RAW 267.4) by LPS treatment lead to their activation, induced morphological changes and significantly increased production of pro-inflammatory cytokine, TNF-α (Fig. 1). However, PE treatment under LPS stimulation inhibited macrophage activation and production of TNF-α both in the mRNA and protein levels (Fig. 1B–D). TNF-α is a critical regulator involved in infection, inflammation and autoimmune disorders (Vassalli, 1992) that is produced majorly by T cells and macrophages in response to a variety of stimulations. Mouse defective in TNF-α production shows resistance to CHS (Pasparakis et al., 1996). Based on the effect of PE in inhibiting macrophage activation and TNF-α production *in vitro* we further tested whether PE treatment also can modulate CHS that is manifested by a network of pro-inflammatory cytokines and by IgE response. CHS is type of common skin hypersensitive response which can be dissected into two stages: sensitization and elicitation phase (Watanabe et al., 2002). The initial stage is the recognition and uptake of the antigen by Langerhan's cells, which become activated and migrate to the skin draining lymph nodes and present the processed antigen to the residual T cells. Elicitation phase ensues following repeated exposure to the same antigen. APCs are a major determinant to mount an effective CHS response (Kripke et al., 1990). In CHS, the local recruitment of Langerhan's cells and their subsequent maturation require the secretion of TNF-α and IL-1β, which are also produced by the Langerhan's cells and macrophages. Upon exposure to the antigens, cells mature and create a cytokine milieu dominated by pro-inflammatory Th1 type, which facilitates induction and exaggeration of CHS (Watanabe et al., 2002). Following the exposure of skin to reactive antigens, keratinocytes and Langerhan's cells at the epidermis of the skin are activated and secrete pro-inflammatory cytokines such as IL-1β and TNF-α that are both immune and inflammatory mediators (Wang et al., 2003). Increased pro-inflammatory cytokines further induce maturation of Langerhan's cells and activate their migratory capacity to the local lymph nodes. Langerhan's cells present processed antigens to the T cells in pro-inflammatory cytokine milieu, which leads to activation, differentiation and migration of effector T cells at the site of infection and exacerbate CHS progression. Porcine PE treatment may inhibit activation of antigen presenting cells (APCs) including macrophages, which led to further down-regulation of pro-inflammatory cytokines (Fig. 6). Does PE itself directly modulate effector function of T cells without modifying APC function? To test this possibility we treated $CD4^+$ T cells with porcine PE and then tested the levels of pro- or anti-inflammatory cytokines. Porcine PE treatment did not induce any significant changes in the tested cytokine levels (data not shown). In human placenta extract studies, glutamate present in human PE showed a beneficial wound healing effect by acting as a chemo attractant to attract neutrophils at the site of infection and helped clearing the infection by phagocytosis (Gupta and Chattopadhyay, 2008). TGF-β and FGF are pivotal

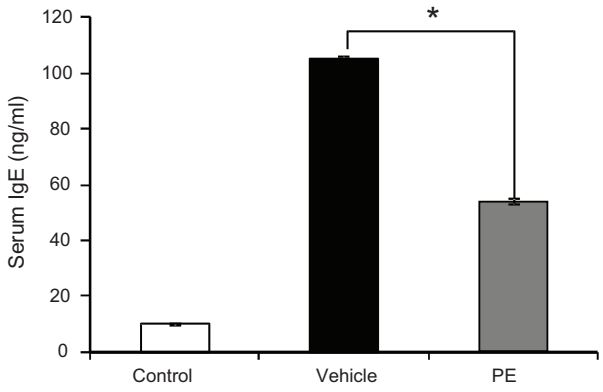

Fig. 4. Effect of porcine placenta extract on the serum IgE levels. Blood was obtained from tail vein of mouse and total serum IgE was measured by ELISA kit (BD Biosciences) with serum diluted 200-fold and absorbance was measured at 490 nm and plotted. Error bars indicates SD. One (*) asterisk indicates $p < 0.05$. Data are representative of three independent experiments.

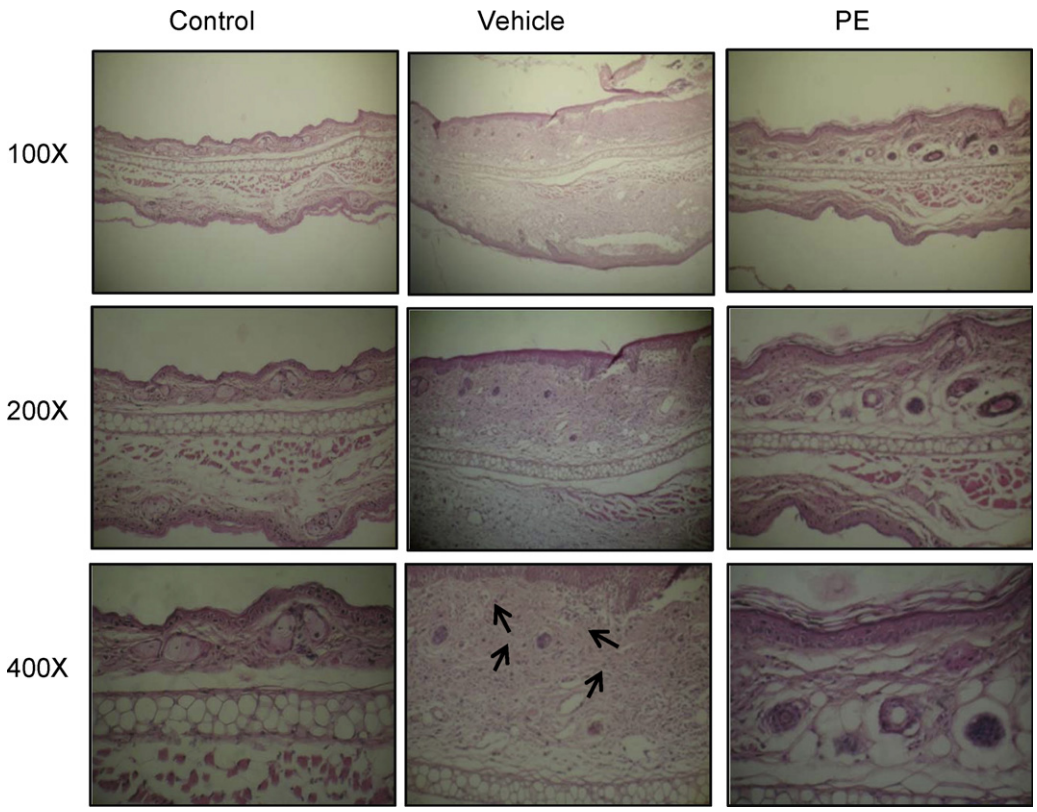

Fig. 5. Histological examination of ear sections of CHS-induced mice following porcine placenta extract treatment. Ears excised from each group of mouse were fixed with 4% paraformaldehyde for 16 h and embedded in paraffin. Paraffin embedded ears were sectioned into 6 μm thin sections and stained with hemotoxylin (Sigma) and eosin (Sigma) (H&E). Infiltrated lymphocytes, thickening of the epidermis were observed under microscope and photographed. Black arrows indicate the increased infiltration of lymphocytes in the inflamed and thickened epidermis of the vehicle-treated group.

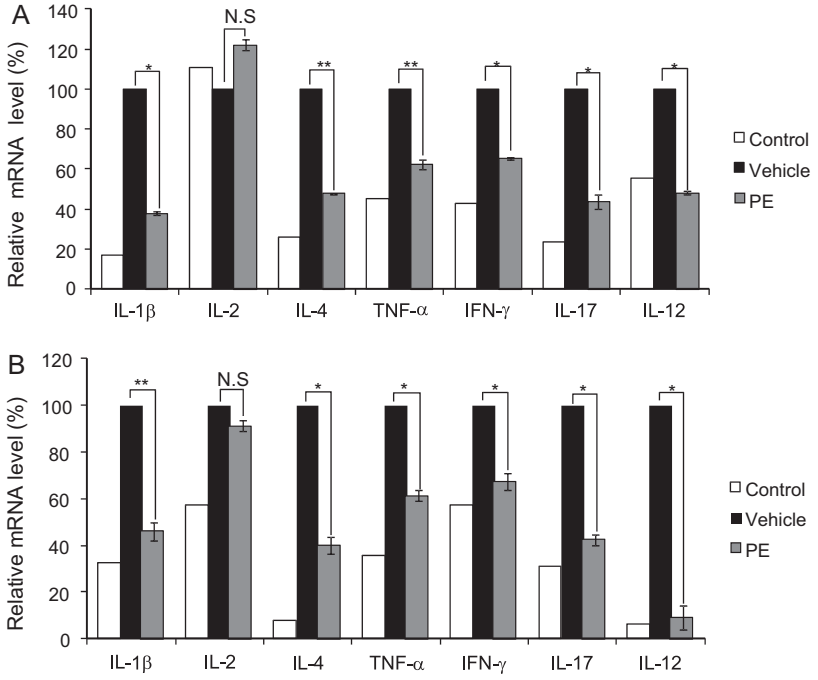

Fig. 6. Effect of porcine placenta extract on the expression of pro-inflammatory cytokines. (A) Single cell suspended total draining lymph node cells and (B) $CD4^+$ T cells were stimulated for 2 h with PMA and ionomycin. Total RNAs were isolated from each sample and reverse transcribed to obtain cDNA. Relative expression level of the cytokines in the experimental group was compared with control group by quantitative real-time PCR. Results are expressed as relative expression levels by taking the expression of the gene in vehicle group as 100%. Error bars indicates SD. One (*) or two (**) indicate $p < 0.05$ or $p < 0.005$, respectively. Data are representative of three independent experiments.

growth factors involved in tissue repair. Placenta extract is a store house of several growth factor cytokines. A report by Wu et al. showed that porcine PE is effective in repairing thermal injury due to the presence of significant amount of FGF and TGF-β (Wu et al., 2003). In this study, to evaluate *in vivo* anti-inflammatory effect of porcine PE in experimental CHS we topically applied the PE in gel form. Topical application of PE decreased ear thickness and progression of ear inflammation and gradually improved clinical symptoms of CHS as evidenced by the inhibition of ear swelling, decreased redness of ears, inflammation, dryness, reduced tissue invasion by lymphocyte, monocytes and neutrophils while improving ear skin texture (Figs. 2–5). Interestingly, in addition to lowering pro-inflammatory immune response, PE treatment decreased IL-4 levels in $CD4^+$ T cells and skin draining lymph nodes (Fig. 6). This result is also well correlated with down-regulated serum IgE levels in PE-treated group compared with control group (Fig. 4). A shift of immune response from Th1 to Th2 type during pregnancy (Lin et al., 1993; Wegmann et al., 1993) protected the developing fetus and prevented spontaneous abortion (Hill et al., 1995; Raghupathy, 1997). However, the role of IL-4 in CHS is still not clear. For example, the neutralization of IL-4 during the challenge phase of CHS aggravated CHS (Gautam et al., 1992). On the other hand, IL-4 knock out mouse shows impaired CHS response to DNCB (Traidl et al., 1999) as evidenced from the significant reduction in magnitude and duration of the CHS response in comparison with wild type mice. STAT6 is a crucial transcription factor of IL-4 signalling pathway (Yokozeki et al., 2000). STAT6 knock out mouse showed a decreased CHS response, which further questions the role of IL-4 in CHS. Regarding the role of IL-10 in CHS, both Traidl et al. and Ferguson et al., confirmed that increased IL-10 production by the inflamed tissue in not necessarily responsible for protection against CHS progression (Ferguson et al., 1994). In our system, PE treatment reduced IL-4 levels without alteration of IL-10 and IL-6 levels (data not shown). Hope et al. reported that IL-6 knock out mouse displays a significant reduction in the acute and second phase inflammatory reaction to oxazolone (OX)-induced CHS (Hope et al., 2000) in C57BL/6 mouse strain. However in our study we used BALB/cJ as our mouse strain and DNCB as the antigen to induce CHS. It is well known that CHS is a complex immune reaction mediated by various cytokines, the expression of which are dependent on the antigen used and strain of mouse used to induce the disease (Wang et al., 2003). Hence this can partly explain the reason as to why we did not detect significant change in IL-6 level in our study.

Report by Kim et al., showed that placenta extract prevents the development of CHS by decreasing IFN-γ while increasing IL-4 and IL-10 levels (Kim et al., 2010). They performed subcutaneous implantation of placenta extract 3 days before sensitization which resulted in a significant inhibition of CHS response. This prior exposure to placenta extract before antigenic challenge probably skewed the system to a Th2 type which in turn protected the mice from developing contact hypersensitivity—a prevention model. Moreover, the first antigenic challenge was on the abdominal region and the secondary sensitization as followed up in the ear lobe in their experiments. On the other hand, in our study, PE treatment was initiated after CHS induction and was continued every day in parallel with repeated challenge with the antigen in the ear lobe (twice weekly) and hence the mechanism of action PE in the two cases is presumably different. We observed that porcine PE treatment significantly down-regulated IL-1β and TNF-α level in CHS model (Fig. 6). IL-1β along with TNF-α is one of the major cytokine that aids in the recruitment of APCs to the skin epidermis sensitized with hapten, which in turn activates the APCs to present the antigen to T cells present in the skin draining lymph nodes (Bennett et al., 2005; Silberberg-Sinakin and Thorbecke, 1980; Wang et al., 1997). PE treatment also reduced other pro-inflammatory cytokine levels such as IL-12, IFN-γ and IL-17 (Fig. 6). These molecules have similarly important roles in the initiation and the progression of CHS as observed in the respective cytokine knock out mouse models (Gorbachev et al., 2001; Wang et al., 2001, 2003). Collectively, down-regulation of IgE levels and pro-inflammatory cytokines by porcine PE treatment may suppress the progression of experimental CHS.

Treatment of placenta extract significantly reduced skin inflammation. What could be the probable underlying action mechanism of PE? Does PE treatment increased $CD4^+$ regulatory T cells, which in turn reduced skin inflammation? Although, we did not estimate the number of $CD4^+$ T cells that have migrated to the peripheral lymphoid organs after PE application, our histology data clearly shows that PE-treatment significantly reduced the number of infiltrated lymphocytes compared with vehicle-treatment (Fig. 5). Moreover, PE treatment also reduced the size of draining lymph nodes compared with vehicle-treatment, which suggest that hyper-immune response elicited during CHS is alleviated by the application of PE. These results suggest that application of PE may decrease the migration of cells to the ear draining lymph nodes. In addition, we measured the expression level of marker molecules related with regulatory T cells such as Foxp3, IL-10 and TGF. However, we could not detect any significant alterations in their expression levels upon PE treatment (data not shown). From our data we therefore concluded, that PE treatment significantly down-regulated the expression levels of pro-inflammatory cytokines such as IL-1β, IFN-γ, TNF-α, IL-12 and IL-17 in total cells of draining nymph node cells, rather increasing regulatory T cells (Fig. 6B). Our *in vitro* data suggest that PE primarily targets the antigen presenting cells (macrophage) (Fig. 1). Recent work by Kim et al., also showed that treatment of placenta extract significantly decreased $CD4^+$ T cells in the PBMC (Kim et al., 2010). However the role of $CD4^+$ T helper (Th) cells versus $CD8^+$ cytotoxic T (Tc) cells in CHS is still unclear. CHS responses were normal in MHC class I (recognized by $CD8^+$ T cells) knock out mice while enhanced in class II MHC (recognized by $CD4^+$ T cells) knock out mice. In addition, amelioration or aggravation of CHS response was not affected by the deletion or over-expression of one prototypical class of such as Th1 or Th2 cytokines, rather affected by both types (Bennett et al., 2005; Pasparakis et al., 1996; Pastore et al., 1996; Saint-Mezard et al., 2004; Silberberg et al., 1976). Apart from T cells and APCs (Langerhan's cells), NK cells have also been found to be critical for hapten specific CHS response. Depletion of NK cells results in impaired CHS response, suggesting a role of NK cells in CHS (O'Leary et al., 2006). However the exact action mechanism still remains unknown. The possible mechanism indicated by another group is that the NK cells are the first infiltrating cells in the hapten challenged sites and the IFN-γ produced by these NK cells serve to introduce the primed T cells to the inflamed antigen challenged sites (Kaneko et al., 2003). In our study, we showed that treatment of PE down-regulated the levels of proinflammatory cytokines IL-1β, IFN-γ, TNF-α, IL-12 and IL-17, indicating a participation of different types of immune cells in the inflamed tissue draining lymph nodes in elicitation of CHS response (Fig. 6B). This is correlated with our *in vitro* data showing that PE treatment inhibited LPS-induced hyper-activation of RAW 264.7 cells and consequent production inflammatory cytokine TNF-α (Fig. 1). Based on our study, PE may primarily target the APCs which thereby create a cytokine milieu that prevents hyper-recruitment of potential CHS mediators including T cells to the inflamed ear-draining lymph nodes. PE may exert its beneficial effect by decreasing the infiltration of major types of immune cells (including $CD4^+$ and $CD8^+$ T cells, DCs, LCs, macrophages and APCs) to the inflamed ear draining lymph nodes.

CHS is a complex hyper-immune reaction that involves the multilayered interaction between various cell types and hence the treatment of the disease requires more than one compound that is essentially available in extracts. Recent report showed that the

compound cyclo-trans-4-L-hydrocypyrolyl-L-serine, isolated from PE, has beneficiary effect to ameliorate CHS (Kim et al., 2010). However, the efficacy of this compound is apparent in the later stage of the disease whereas PE is effective from the early stage of the disease. This indicates the presence of other compounds in PE that works in coordination to bring about the beneficial effect of PE in CHS. One explanation by which placenta extract exerts beneficial effect could be that some compounds in the PE could increase the solubility and absorption rate of the effective components, thereby enhancing their bioavailability. In our study the aqueous fraction of porcine PE may exert its immunomodulatory role by down-regulation of proinflammatory cytokines produced by diverse immune cells, presumably by preventing activation of antigen presenting cells (including macrophages).

5. Conclusion

Although beneficial effects of placenta extract from various animals have been noticed for a long time, the definitive action mechanism was not clear. In this study we have shown the potent immunomodulatory function of porcine PE in experimental contact hypersensitivity. Porcine PE treatment inhibited macrophage activation and production of TNF-α *in vitro*. Topical application of porcine PE suppressed CHS progression by down-regulating the key cytokines of CHS pathogenesis such as IL-1β, TNF-α, IL-12 and IL-17. In addition, PE treatment reduced serum IgE levels and IL-4 expression. These results suggest that PE may exert its anti-inflammatory effect by reducing the hyper-activation of antigen presenting cells, which in turn decreased production of inflammatory cytokines by T helper cells and other lymphocytes. Identification of the active compounds responsible for immunomodulatory activity of porcine PE in CHS progression will potentiate its use. Currently, we are in the process of identifying and comparing the effects of the individual components of porcine PE with the whole extract. Although, we still need to define the anti-inflammatory mediators and exact components of the extract responsible for this property, porcine PE does exert a beneficial role in decreasing or modulating the severity of the inflammatory responses associated with CHS and thereafter.

Conflict of interest

Authors do not have conflict of interest.

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