Results

Identification of Growth Factors in Placental Extracts

The porcine and human placenta tissue was extracted with different aqueous solvents and the extracts were identified of growth factors related with wound healing. The presence of growth factors in four extracts of porcine placenta were analyzed by Western blot and determined by ELISA. As shown in Fig.1, TGF-β1 and bFGF were detected in porcine placenta extracts. TGF-β1, which is a 25.0 kDa homodimeric protein, regulates the proliferation and differentiation of cell and ECM synthesis. Basic FGF is one of the first angiogenic factors to be characterized and has been extensively studied. The single-copy human bFGF gene encodes multiple FGF-2 isoforms with molecular weight (MW) ranging from 18 to 24 kDa (Florkiewicz RZ and Sommer A, 1989). ELISA in Table 3, the yield of growth factors, from high to low, is 2 M urea, 1 M $MgCl_2$, 0.1 M NaCl and ddH_2O porcine placenta extracts. It seems that 2 M urea is better extraction solution for placenta extraction.

Placental Extracts Promoted Cell Proliferation

Fibroblasts and keratinocytes are the two major cell types in the dermis and epidermis responsible for wound healing, and therefore are selected to determine if placental extracts affect them in wound healing. To mimic the wound environment, we used cell cultured under very low serum conditions. The presence of small amounts of serum in the culture medium mimics the conditions of the healing adult wounds, where serum factors are more abundant than in normal tissue (Feugate JE et al., 2002). In addition, to ensure that no factors present in serum could influence the results (Rieck W et al., 2001). We designated 0.5% serum or 0.1% BSA as negative control, and 15% serum as positive control in accordance with Wu CH et al. study. To test the effect of placental extracts on cell proliferation, cells were treated with

various concentrations of placental extracts. Cells were seeded in 24-well plates, and then cells were starved in serum free medium for 18 h. NIH3T3 fibroblasts and HaCaT keratinocytes were treated with porcine or human placental extracts for 20 h and 24 h, respectively. Then, cell was analyzed by MTT assay. This assay determines the number of viable cells in proliferation assay, because the total amount of the formed formazan end product is directly proportional to the number of living cells in culture. As shown in Fig. 2.A and 2.B, all placental extracts promoted NIH3T3 and HaCaT cell proliferation in a dose-dependent manner compared to 0.5% serum as negative control, respectively. The cellular response to H_2O, NaCl, $MgCl_2$ and urea extracts were similar. Among all extracts, the 2 M urea extract of porcine placenta simulated NIH3T3 and HaCaT cell proliferation occurs more quickly than other extracts. Moreover, angiogenesis, formation of new blood vessels from existing endothelium, occurs during wound repair. The process of angiogenesis consists of migration and proliferation of endothelial cell. We also evaluated the effects of placental extracts on CPAE cell. As shown in Fig. 2.C, the response of CPAE cell to placental extracts was similar to NIH3T3 and HaCaT cell. Compared with porcine placental extracts, human placental extract were inferior to porcine placental extracts, but no significantly different.

Placental Extracts Promoted Cell Migration

During wound healing, chemokines are able to chemoattract fibroblasts and keratinocytes, and accelerate their migration. In the skin, wound healing is completed by the migration of keratinocytes from the wound edges to re-epithelialize the open wound bed. Migration of fibroblasts from the area surrounding the wound into the wound site is critical in order for remodeling of the ECM and for wound contraction to occur. In addition, the process of angiogenesis consists of migration and proliferation of endothelial cell. To determine whether placental extracts regulate keratinocyte and endothelial cell motility, we used scratch assay considered to be an in vitro model for cell migration occurring during wound healing (Wells

A, 2000). Confluent monolayers of HaCaT and CPAE cells were wounded with a P-200 pipette tip, and then treated with four extracts of porcine placenta in 0.1% BSA serum-free culture medium for 24 h. As shown in Fig. 3.A and 3.B, some HaCaT and CPAE cells had migrated into the wound area, outlined by the straight mark. In control, the 0.1% BSA serum-free culture conditions contributed to the low migration activity. Application of porcine placental extracts and 10% or 20% serum served as positive control to the culture medium increased the wound-induced movement of HaCaT and CPAE cells, respectively. Scratch closure after 24 h was enhanced by treatment with porcine placental extracts. All extracts stimulated keratinocytes and endothelial cells migration. Among all extracts, the 2 M urea extract and 1M $MgCl_2$ of porcine placenta apparently simulated cell migration more than other extracts. Simultaneously, we observed HaCaT and CPAE cell proliferation in scratch assay.

Placental Extracts Mediated Cell Adhesion

The adhesive contacts between cells and the ECM are important for cellular migration and proliferation, both of whom were significant parameters in wound healing, as well as the initiation and formation of intercellular adhesion. To study whether there is an interaction between cells and placental extracts, we used cell adhesion assay. Cells were harvested and allowed to adhere on precoated with various concentrations of different porcine placental extracts and 5 μg/ml fibronectin served as standard plates. As shown in Fig. 5.A, B and C, placental extracts were supportive in mediating adhesion of NIH3T3, HaCaT and CPAE cells, respectively. All extracts mediated cell adhesion in a dose-dependent manner. Among all extracts, the mediated cell adhesion ability of 0.1 M NaCl extract of porcine placenta was superior to other extracts, whereas 2 M urea extract of porcine placenta was inferior. However, our data showed that 2 M urea extract is a better extraction solution in cell proliferation and migration assay. We suggest that some cell adhesion proteins in placental extracts that mediate

various aspects of cellular associations with ECM through a variety of integrin–ligand interactions. The effects of porcine placental extracts on wound healing were through cell and adhesion protein in placental extracts interactions or cell and growth factors in placental extracts interactions (Fig. 6). Therefore, we presume that 2 M urea extract promoted cell proliferation and migration probably because of higher content of growth factors principally.

RT-PCR evidence of gelatinase mRNA Expression

Because MMPs, once activated, are collectively capable of degrading the complete ECM, it is important that the activity of these enzymes is kept under tight control. The activity of MMPs is controlled at the following three levels: transcription, activation of the latent proenzyme, and inhibition by their endogenous inhibitors, the TIMPs (Creemers EE et al., 2001). Total RNA was extracted from HaCaT cells and 5 μg of total RNA were subjected to RT and amplification by PCR. As shown in Fig. 7, PCR products were clearly observed. MMP-1 and MMP-9 had no obviously change in transcription level. Porcine and human placental extracts didn't regulate these MMPs at the level of mRNA.

Placental Extracts Promoted MMP Production

Zymography on SDS-gelatin was used to determine whether HaCaT cells secreted gelatin-degrading MMPs. Both active and latent forms of MMP-2 and MMP-9 were measured using zymography, because the zymography process (a denatureation of the enzyme for electrophoresis and a renaturation before incubation for activity determination) activities the various proenzyme forms so that they can be measured (Birkedal-Hansen H, et al., 1982). The proenzyme forms of gelatinases display proteolytic activity on zymography gels and can be distinguished from the activated forms by their migration properties. As shown in Fig. 8 and 9, gelatinase activities were detected in three main forms: two major bands at 92 and 72 kDa produced by pro from gelatinase B (MMP-9) and pro from gelatinase A (MMP-2),

respectively; a minor band 68 kDa corresponding to active form gelatinase A, and were increasingly with time course. Time-course experiments in zymography showed that exposure of cells to dose 50 μg/ml placental extracts for 48 h yielded the greater response (Fig. 8). We toke 48 h as time point, and examined placental extracts in MMPs expression. Treatment with 50 μg/ml porcine placental extracts induced high level of secretion of the gelatinolytic enzyme compared with the control. Moreover, culture medium in the presence of porcine placental extracts activated 72 kDa gelatinase into smaller band of 68 kDa. As shown in Fig. 9.A and B, porcine and human placental extracts stimulated MMP-2 expression and activation. Quantitative analysis showed enhanced levels of latent form of MMP-9 and latent and active forms of MMP-2 after treatment of placental extracts. Active MMP-2 was increased with 50 μg/ml H_2O, NaCl, $MgCl_2$ and urea porcine placental extracts about 1.7, 1.5, 2.4, and 2.5-fold increase, respectively, compared with the serum-free medium control. And, active MMP-2 was increased with 50 μg/ml H_2O, NaCl, $MgCl_2$ and urea human placental extracts about 1.7, 1.7, 1.9, and 2.1-fold increase, respectively, compared with the serum-free medium control. The ability to active MMP-2 in human placental extracts and porcine placental extracts is no significant different. 2 M urea extract of either porcine or human placenta is still the better extraction solution. Our data showed that both porcine and human placental extracts up-regulated these MMPs at the level of protein synthesis.