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Cell adhesion by aqueous extract of human placenta used as wound healer

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Aqueous extract of human placenta, used as wound healer, has shown significant cell adhesion property on mouse peritoneal macrophages and P_{388D1} cultured macrophage cell line. This property was offered primarily by fibronectin type III like peptide present in the extract and is comparable to fibronectin on a molar basis. The peptide induce adhesion of cell through cell surface receptors having $K_d = 2.8 \pm 0.9 \times 10^{-5}$ *M* suggesting weak binding. This is in support of integrins receptors that typically exhibit low affinities. Cell adhesion was partially inhibited by Arg-Gly-Asp (RGD) peptide, anti-β_1 integrin suggesting that integrin β_1 receptors have roles to play in the process.

Keywords: Anti-β_1 integrin, Cell adhesion, Human placental extract, RDG peptide.

Derived from folk knowledge, human placental extract has long been used in therapeutic purposes like wound healing, ophthalmology, infertility, apoplexy, epilepsy and others. Being a link between mother and fetus, it provides all nutrients and protective shield to the baby. It is a rich source of damage repairing agents like hormones, proteins, enzymes, glycosaminoglycans, nucleic acids etc and thus the evolution is rational[1]. Active components present in aqueous extracts vary depending on the method of its preparation. In India, such extract is used as licensed drug in burn injuries, chronic wounds and surgical dressings[2,3]. Applications of similar extracts are known in the treatment of chronic wounds, arthritis and melanogenesis in other countries[4-6].

It is noteworthy that therapeutic potency of placental extract has been confirmed by practicing physicians over the years, components present in it and their roles towards wound healing are not defined. Recently, we have initiated evaluation of such extract manufactured indigenously as drug. That includes demonstration of high degree of batch consistency[7], presence of biologically active NADPH[8] and human fibronectin type III like peptide (FN-peptide)[9]. In addition NO induction and antibacterial property of the extract against different pathological and drug resistant strains has been shown, thus supporting at least partial prevention of secondary infections in chronic wounds[10,11]. Here, we report that one or more components of the extract bind to the surface of mouse peritoneal macrophage and cause morphological changes towards adhesion and spreading of the cells.

Materials and Methods

Reagents—The drug house M/s Albert David Ltd., Calcutta, India, supplied human placental extract as sold under the trade name 'Placentrex'. Method of preparation of the extract holding manufacturer's proprietary protocol has been described earlier[8]. To ensure biological safety, each extract was tested for HIV antibody and hepatitis B surface antigen. Further, the sterilization procedure holds sufficient margin to destroy most resistant spore-producing species like *Clostridium tetani*.

RPMI media, Fetal Bovine Serum (FBS), Pen-Strep, peroxidase conjugated goat anti-mice IgG and 3,3′-diaminobenzedine tetrahydrochloride were from Life Technologies, Bangalore, India. Human cellular fibronectin, Arg-Gly-Asp (RGD) peptide, anti-β_1 integrin, fluorescence isothiocyanate (FITC) and benzoylated dialysis membrane (cut-off range 2 kDa) were from Sigma (USA). Macrophage cell line P_{388D1} was kindly provided by Dr. Syamal Roy of our institute. Peritoneal macrophage cells were isolated from BALB/C strain mice obtained from in-house

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breeding facility. Electrophoretic, staining and other reagents were of highest grade available locally. Protein estimation was done after modified method of Lowry with BSA as reference[12].

Peritoneal macrophage cell isolation—Macrophage cells were pulled out from peritoneal cavity of BALB/C mice sacrificed after 2 days from injecting 1 ml of 4% solution of starch in PBS, *p*H 7.4[13]. After three times washing with PBS, cells were suspended in RPMI medium at a concentration of 3×10^5 cells/ml. The animals were sacrificed by cervical dislocation and destroyed in incinerator as par animal ethics guideline of this institute.

Fractionation of proteins and peptide—Placental extract as supplied was concentrated 50-fold in vacuum desiccators over NaOH pellets at ambient temperature and was centrifuged whereby a fat layer and insoluble materials were separated. Proteins/peptides from the recovered yellowish liquid, referred as 'fat free extract', were fractionated using three procedures. First, the liquid was dialyzed against water to remove small bio-organic molecules, salts and peptides of <2 kDa ('dialyzed sample'). Second, 100 µl of 'fat free extract' was passed through 1-ml Sephadex G-75 (fractionation range 3-80 kDa) 'spin column' equilibrated with water in multiple batches to remove proteins and peptides well below 70 kDa ('spin column eluant')[14]. The eluant contains considerable amount of FN-peptide of Mw 7.2 kDa because the peptide has a tendency to aggregate[9]. Third, the 'fat free extract' was diluted 10-fold with water of which 1-ml was applied to Centricon Nylon membrane (YM-10, Millipore, cut off limit 10 kDa). Filtration was done under mild centrifugal force (avg. 2000 g). The filtrate was dialyzed using benzoylated dialysis membrane against water with several changes. This preparation yields purified FN-peptide that has been previously identified and characterized[9]. It has been confirmed using catalase (Mw 240 kDa) that to the limit of detection, the membrane was capable of retaining the protein by 100% and therefore, possibility of contamination of fibronectin (Mw about 550 kDa) in FN-peptide remained insignificant.

Cell attachment and spreading assay—For cell adhesion assay, 1ml of placental extract as supplied or its fractions at 0.3 mg/ml was coated onto tissue culture Petridishes 35 mm diam. The dishes were washed 3 times with PBS to remove unbound components of the extract before cells were added. Peritoneal macrophage cells (3×10^5/ml suspended in RPMI in presence and absence of 10% FBS) were seeded on the coated petri dishes with 1ml of the solution. Cells were allowed to adhere for 30 min at 37°C followed by fixing with 0.5 ml of 0.25% aqueous glutaraldehyde per dish. Then it was washed 3-4 times with distilled water and cells were stained with 0.25% crystal violet in 20% methanol for 15 min at ambient temperature. After washing the cells with PBS, the dye was solubilized and extracted with 0.1 *M* Na-citrate, *p*H 4.2 containing 50% ethanol. Cell adhesion was quantified by measuring turbidity at 600 nm[15,16]. Similar experiments were performed after incubating the peritoneal macrophage cells (3×10^5 cells/ml) with RGD (0.5 mg/ml), anti-β_1 integrin (100 µg/ml), and EDTA (15 mM) at 4°C for 90 min.

Cell attachment and spreading assay were performed similarly using cells from peritoneal and cultured macrophage cell line P_{388D1}. Palate cells were suspended at 3×10^5 cells/ml in RPMI medium and seeded on petridishes coated with FN-peptide (50 µg/ml), bovine serum albumin (20 µg/ml) or PBS. After incubation for 30 min at 37°C, cells were fixed with 0.5 ml of 0.25% glutaraldehyde and were stained with Coomassie Blue dye (1 mg/ml in methanol:acetic acid:water = 45:10:45). Desalting was done with water and cells were viewed with fluorescence microscope (Olympus) after air-drying[16].

Since bovine serum contains 0.2-0.4 mg/ml of fibronectin, cell adhesion were carried out both in presence and absence of 10% FBS to check the effect of the adhesive molecule. In presence of FBS, effect of FN-peptide could not be demonstrated clearly because of high background. In its absence, cell adhesion by FN-peptide was prominent against low background.

Raising of antibody—Antibody against FN-peptide was raised in BALB/c mice by injecting subcutaneously 25 µg of the peptide in 100 µl of PBS after emulsifying with equal volume of Freund's complete adjuvant. Because of low antigenecity of peptides, the process was repeated 5 times after every 15 days using Freund's incomplete adjuvant. Blood was drawn from tail vein of the animals after 15 days from last application of the antigen and serum was collected[17].

Immuno-blotting—After electrophoresis in 15% SDS-PAGE of FN-peptide, the peptide and its aggregated forms were transferred to nitro-cellulose

membrane using an electro-transfer apparatus (mini gel apparatus; Genei, India) at 100 mA for 2 h. To reduce background, membranes were incubated overnight in blocking buffer (100 m*M* TBS; *p*H 7.0 containing 2% BSA) at 4°C followed by washing once with washing buffer (0.15% Tween-20 in TBS, *p*H 7.0). Then the membrane was incubated for 1 h with the raised antibody at 1:1000 dilution with the washing buffer under constant shaking followed by washing twice each for 20 min duration. The membrane was further incubated for 1 h with constant shaking in 1:3000 dilution of peroxidase-conjugated goat anti mouse IgG followed by washing thrice with distilled water. Finally, immunological cross reactivity was revealed from color development after treatment for 10 min with 5 m*M* of 3,3′-diamino-benzedine tetrahydrochloride (Sigma Immuno-chemicals) in PBS containing 0.02% of H_2O_2[18].

Peptide binding assay—Binding of FN-peptide to macrophage cell was quantified using FITC as fluorescence probe. FN-peptide (1 mg/ml) in 0.25 m*M* Na-carbonate, *p*H 8.5 was added to celite-FITC (0.5 mg celite powder/mg of peptide) with immediate vortexing and the mixture was incubated at ambient temperature for 30 min as par manufacturers protocols[19]. Unbound FITC was removed by passing the peptide through 'Sephadex G-10' spin column[14]. Assuming that 95% of the peptide was recovered and no free fluorophore were eluted, concentration of the labeled peptide in subsequent steps were considered to be proportional to their emission intensity (ex: 492 nm; em: 515 nm). Peritoneal macrophage cells (3×10^5 cells/ml) were incubated with graded concentration of tagged-peptide for 90 min in PBS containing 1% BSA. Cells were also pre-incubated for 30 min in PBS containing 1% BSA for blocking non-specific protein binding sites. After centrifugation at 2000 rpm for 3 min, supernatants was decanted off. Binding studies were conducted at 4°C, since at this temperature cellular processes were slow and receptor internalization were negligible[20]. Precipitated peptide tagged cells were suspended in 1-ml of PBS and bound peptide was quantified using emission intensity.

Results

Cell adhesion by placental extract—Adhesion of macrophage cell by placental extract as supplied and its different fractions was estimated, where PBS served as control (Fig. 1). Ability of adhesion has been expressed in % relative to purified FN-peptide on weight basis, where adhesion was maximum. The supplied extract showed marginal enhancement of cell adhesion over control (20 versus 16%). Presence of lipids in the extract probably inhibited cell attachment with the coated petridishes *in vitro*. Considerable increase of activity, between 80-93% was observed with 'fat free' extract and the 'dialyzed' fraction. The 'spin column' eluent offered 40 ± 10% of adhesion with 50 ± 5% recovered proteins; the result suggested activity of higher molecular weight components of the extract. Considering very low protein content of the extract and aggregate prone character of FN-peptide, its multimers seem to offer cell adhesion.

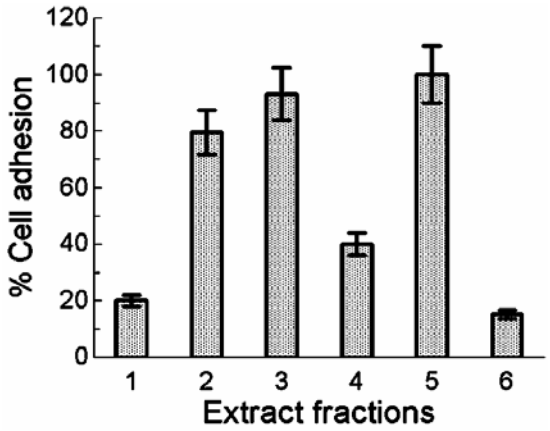

Fig. 1—Relative cell adhesion (%) measured by coating 1-ml of different fractions of placental extract containing 0.3 mg of protein/peptide. [Lane 1-Extract as supplied; Lane 2-Fat free fraction of the extract; Lane 3-Dialyzed sample; Lane 4-'Spin column' eluent; Lane 5-FN-peptide and Lane 6-Cell seeded on PBS coated dishes serving as control. Number of batches tested was 4 and from each batch, 2 sets were prepared where variation of results are represented as means ± SD]

Peptide aggregates—SDS-PAGE of the placental extract after trichloroacetic acid precipitation showed a major component of 6-7 kDa together with components of 60-90 kDa of low abundance as reported earlier[9]. Purification of the peptide using Centricorn YM-10 rendered it free from high Mw proteins. Almost complete conversion of the peptide (1 mg/ml) to multimers of variable Mw after storage for 10 days at 4°C in water was observed by SDS-PAGE (Fig. 2A). Origin of the aggregates from FN-peptide was confirmed by immuno-blot using mouse antibody raised against the purified peptide (Fig. 2 B). Human cellular fibronectin, serving as control, reacted strongly with the antibody. During an intermediate storage period of 2 days, the antibody cross-reacted with aggregates of 50-70 kDa along

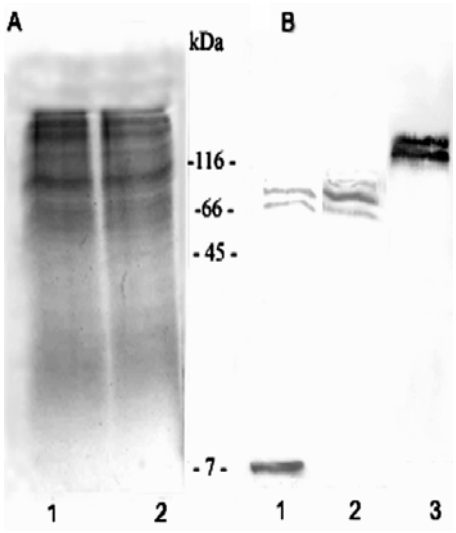

Fig. 2—(a)-SDS-PAGE (15%), [Lane 1 and 2-FN-peptide, 20 and 25 μg, after storage for 10 days at 4°C in water];. (b)-Immuno-blot using antibody of FN-peptide, [Lane 1-Purified FN-peptide (25 μg), after storage for 2 days at 4°C in water; Lane 2-FN-peptide (25 μg), after storage for 10 days at 4°C in water; and Lane 3-Human cellular fibronectin (30 μg)].

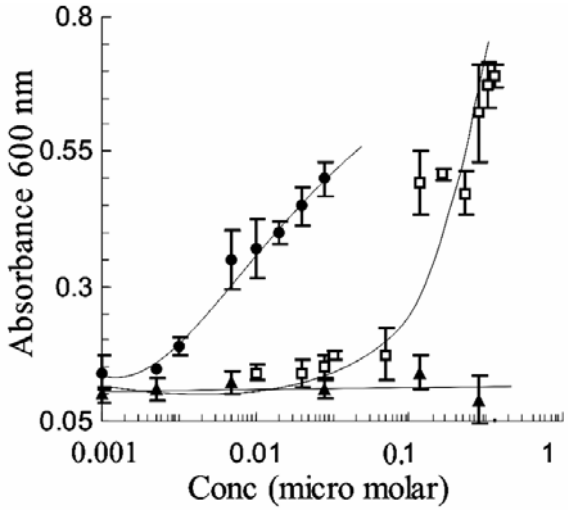

Fig. 3—Adhesion of peritoneal macrophage cells on Petridishes coated with increasing concentration of (●) human fibronectin; (□) purified FN-peptide; and (▲) bovine serum albumin. Adhered stained with 0.25% crystal violet in 20% methanol for 15 min was solubilized and extracted with 0.1 *M* sodium citrate, *p*H 4.2 containing 50% ethanol and quantified by measuring absorbance at 600 nm. Number of batches tested was 4 and from each batch, 2 sets were prepared where variation of results was represented as means ± SD.

with 7.2 kDa FN-peptide. The same fraction, after storage for 10 days showed bands in the range of 50-70 kDa with almost complete disappearance of 7.2 kDa band. Neither aggregate formation nor its cell adhesion property was affected in presence of β-mercaptoethanol suggesting absence of cystein bonds in the process.

Cell adhesion and spreading by FN-peptide—Based on the results described so far and background literature[21,22], cell adhesion and spreading activity of FN-peptide is indicative. It was directly tested on mouse peritoneal macrophage cells coated on dishes where human cellular fibronectin and BSA served as positive and negative controls respectively. Cells adhere efficiently to human fibronectin and FN-peptide, but not with BSA. It is illustrated in Fig. 3, where cell adherence has been presented against molar concentration of protein/peptide in log scale because of wider difference in Mw of fibronectin (550 kDa) and FN-peptide (7.2 kDa). On molar basis FN-peptide was 50-60-fold less effective than full-length fibronectin. This is expected because of multiple type III repeats containing cell binding domain (CBD) with RGD sequence and the synergy site presenting the Pro-His-Ser-Arg-Asp (PHSRN) in full-length fibronectin. This matches nearly to 76.3, the ratio of Mw of whole fibronectin and FN-peptide. A similar ratio has also been reported in case of adhesion of rat aortic smooth muscle cells (RASMCs) by fibronectin 1st type III repeat compared to whole fibronectin molecule[15,23].

FN-peptide not only attaches cells on coated dishes, but also affects their morphology at early times of plating. Peritoneal macrophage cells plated onto FN-peptide coated dishes remained attached and spread by 10-15 fold greater numbers through large surface area (Fig. 4A). On the other hand, cells seeded onto PBS and BSA coated plates neither showed any morphological changes nor any preference to attached cells. Same results were obtained with macrophages P_{388D1} (Fig. 4B). Cell attachment and spreading was remarkable in case of FN-peptide coated dishes, with typically containing numerous lamellipodia per cell as compared to control plates. A single peritonial macrophage cell has been magnified where it shows almost 2-fold rise in surface area and spreading (Fig. 4C).

FN-peptide binding on cell surface—Preliminary experiments showed that peritoneal macrophage adhere to FN-peptide immobilized on plastic surfaces but not to BSA-coated surfaces. Under fluorescence microscope, it has been observed that all FITC tagged FN-peptide remained localized on the surface of the cell (result not shown). Thus, it is likely that

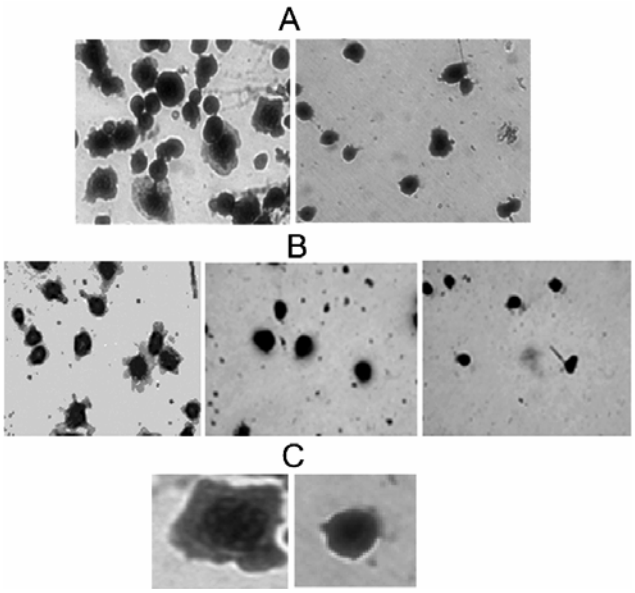

Fig. 4—Macrophage cell adhesion and spreading on tissue culture petridishes. (a)-Mouse peritonial macrophage cells [left panel: by FN-peptide (0.1 mg/ml) and right panel: PBS]; (b)-P_{388D1} macrophage cell line on Petridishes coated with [left panel: FN-peptide (0.1 mg/ml), middle panel: BSA (0.2 mg/ml) and right panel: PBS]. Photograph of cells have been taken at a magnification of 400 X. (c)-A single peritoneal macrophage cell [left panel: on FN-peptide coated dish has been again magnified (2-fold) to compare the extent of cell spreading with respect to, right panel: PBS as control].

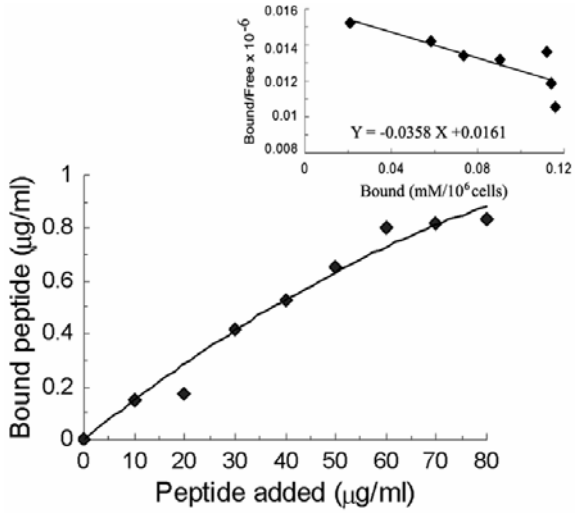

Fig. 5—Peritonial macrophage cells (3×10^5 cells/ml) were incubated with graded concentrations of FITC-tagged purified peptide for 90 min at 4°C in PBS containing 1% BSA solution (n = 3). Bound peptide was quantified by measuring fluorescence emission of FITC (ex: 492 nm; em 515 nm) ($R^2 = 0.9742$). (inset) Derived Scatchard plot yielded overall K_d of $2.8 \pm 0.9 \times 10^{-5}$ *M* at 25° C ($R^2 = 0.9738$).

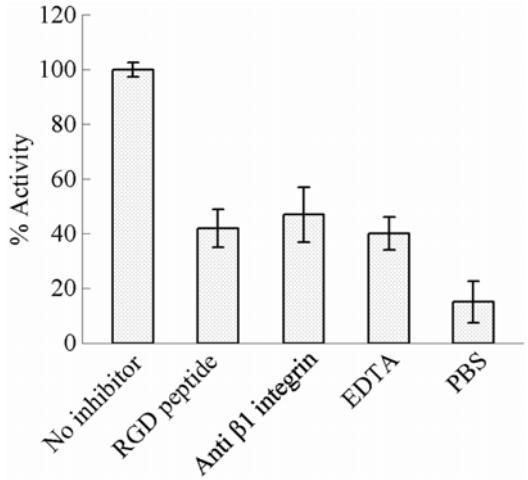

Fig. 6—Peritonial macrophage cell adhesion in relative % on FN-peptide coated dishes after pre-incubation of the cells for 90 min at 4ºC with integrin β_1 blocking inhibitors [Lane 1-Without inhibitor; [Lane 2-RGD peptide (0.5 mg/ml); [Lane 3-Anti β_1 integrin (100 µg/ml); [Lane 4-EDTA (15 m*M*); and [Lane 5-PBS coated plates taken as control. Number of batches tested was 4 and from each batch, 2 sets were prepared where variation of results are represented as means ± SD].

FN-peptide induce adhesion of cell through cell surface receptors. Corresponding binding affinity was determined after incubating cells (3×10^5/ml) with 0-80 µg/ml of FITC-tagged FN-peptide in presence of 1% BSA as non-specific receptor blocker. Dependence of bound to total peptide added had been shown in Fig. 5. Derived Scatchard plot was linear yielding a dissociation constant $K_d = 2.8 \pm 0.9 \times 10^{-5}$ *M* (n = 3) (Inset, Fig. 5). Higher concentration of the peptide towards saturation of cell surface was avoided because of its low biological significance. Result is in support of integrins receptors that typically exhibit low affinities ($K_d = 10^{-6}$ - 10^{-8} M) for their ligands as compared to high affinities ($K_d = 10^{-9}$ - 10^{-11} M) of typical hormone receptors. It is believed that innumerable integrin receptors compensate their weak interactions that allow a cell to bind firmly by anchoring to extracellular matrix proteins [21].

Role of integrin in cell adhesion—Attachment of cells was further analyzed by integrin as potent adhesive receptors. Cells were incubated for 90 min at 4°C with integrin-blocking reagents like RGD peptide (final concentration, 0.5 mg/ml), anti β_1 integrin (50 µg/ml) and EDTA (15 m*M*). Results indicated that cell adhesion was reduced to 42-47% by β_1 integrin receptor blockers (Fig. 6). It is indicative that integrin receptors responsible for cell adhesion are similar to human fibronectin type III[22]. Heparin and heparan sulphate have been used to verify the roles of cell

surface proteoglycan heparin as a potent adhesive receptors where 10-22% inhibition was observed. However, when integrin receptor inhibitor and cell surface proteoglycan receptor inhibitor were applied together, inhibition was marginally raised to 12-18% (results not shown). In fibronectin, the RDG sequence and the heparin-binding site are distant apart. Since FN-peptide has been derived from fibronectin, it is unlikely that it could accommodate both the sites within 7.2 kDa.

Discussion

Under physiological and pathological conditions, macrophages participate in modulating extra cellular matrix (ECM) turnover directly by secreting matrix metalloproteases (MMPs) and their inhibitors[25,26] or indirectly by releasing cytokines[27]. This is an essential process of proper wound healing[28]. Adherence to fibronectin, an ECM component, promotes migration and phagocytosis of cells. It modulates the expression of inflammatory cytokines like interlukin-1, tumor necrosis factor-α and macrophage colony stimulating factor (M-CSF)[29,30]. These functions of fibronec-tin are primarily mediated by cell surface receptor $\alpha_5\beta_1$ integrin, which recognizes the cell binding RGD sequence present in its type III module[31]. As a result, adhesion and spreading of cells occur[22,32].

Since placental extract under investigation contains substantially high amount of FN-peptide, it was logical to investigate whether the extract and the FN-peptide could demonstrate cell adhesion and spreading similar to human fibronectin [15]. The peptide and its aggregated form indeed showed these properties (Figs 3-5). The extract contains lipid that interferes attachment of cells on petridishes. The lipid might be beneficial for efficient distribution of FN-peptide through tissue system[33]. Formation of aggregate appears to be a result of laboratory manipulation and may not be present in the drug[34]. The purified FN-peptide has shown binding to the cell-surface (Fig. 5). It has been shown that cell adhesion is mediated through integrin β_1 receptors as adhesion has been partially inhibited by integrin blocking agents like RGD peptide, anti- β_1 integrin and EDTA (Fig. 6). This is one of the major mechanisms for cell adhesion and wound healing by fibronectin type III[15,21].

Many isoforms of fibronectin are formed in cells. Alternative splicing presumably allows a cell to produce the type of fibronectin that is most suitable for the needs of the tissue. Most of the splicing sites are present in mRNA precursor of type III domain[21,35]. The pattern of fibronectin RNA splicing in the early embryo is different from that seen later in development. When an adult skin is injured, the pattern of fibronectin RNA splicing in the base of the wound switches back to the pattern seen in early development[36]. These observations suggest that the forms of fibronectin produced in the early embryo and in wound healing are especially appropriate for promoting the cell adhesion, migrations and proliferation required for tissue development and repair[22,36,37].

Placenta being a supplier of nutrients and protective shield, it is a rich source of organic compounds, lipids, carbohydrate, nucleic acid, peptides and proteins. The ligands often modulate the activity of the macromolecules by changing its surroundings environment[38,39]. It is to be seen whether fatty acids and carbohydrates remain bound to FN-peptide and regulate its cell adhesion property.

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