Thesis entitled

Isolation and characterization of Placental Epap-1 Secreting Endothelial cells and an analysis of anti-viral activity of recombinant Epap-1.

Submitted for the award of

DOCTOR OF PHILOSOPY

by

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This is to certify that I, ***Pelluru Dheeraj*** have carried out the research work embodied in the present theses entitled "**Isolation and characterization of Placental Epap-1 Secreting Endothelial cells and an analysis of anti-viral activity of recombinant Epap-1"** and submitted for the degree of Doctor of Philosophy was accomplished for the full period prescribed under PhD ordinances of the University, under the supervision of Dr. Anand K. Kondapi, in the Department of Biochemistry, School of Life Sciences, University of Hyderabad.

I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award of research degree of any University.

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ABBREVIATIONS

µg	: microgram
µl	: microlitre
µM	: micro molar
AIDS	: Aquired Immunodeficiency syndrome
ALP	: Alkaline Phosphotase
ATP	: Adenosine 5' triphosphate
BCIP	: 5- bromo – 4 – chloro – 3 indoyl phosphate
Bis-acrylamide	: N,N'- methylene –bis –acrylamide
BSA	: Bovine serum albumin
CCR5	: cystein-cystein linked chemokine receptor 5
CD	: cluster of differentiation
cDNA	: complementary DNA
CXCR4	: cystein-x-cystein linked chemokine receptor 4
DMSO	: Di methyl sulphoxide
DNA	: deoxy ribonucleic acid
DNase I	: deoxy ribonuclease
DTT	: Dithiothreitol
EDTA	: Ethylene di amine tetra acetic acid
ELISA	: Enzyme linked immunosorbent assay
Epap-1	: Early pregnancy associated protein
EtBr	: Ethidium bromide
FCS	: Fetal calf serum
Fig	: Figure
hCG	: human chorionic gonadotrophin
HIV-1	: Human Immunodeficiency virus
HLA	: human leukocyte antigen
IFNγ	: interferon gamma
Ig	: immunoglobulin
IL	: interleukin

Kbp	: kilo base pairs
KDa	: Kilo daltons
LB	: Luria- Bertani
MAb	: Monoclonal antibody
Met	: Methionine
Met.tRNAi	: initiator transfer RNA
mg	: milligram(s)
min	: minutes
ml	: millilitre
Mr	: Marker
mRNA	: messenger RNA
MTP	: Maternally terminated placenta
NBT	: Nitro blue tetrazolium
ng	: nanogram
NK	: natural killer cells
NSI	: non syncytium inducing
p.i.	: post infection
PAGE	: Poly acrylamide gel electrophoresis
PBMCs	: poly blood mononuclear cells
PCR	: Polymerase chain reaction.
PBS	: Phosphate buffer saline
RNase	: Ribonuclease
rpm	: rotations per minute
RT	: reverse transcriptase
SDS	: sodium dodecyl sulohate
TEMED	: N',N',N',N'- tetra methyl ethylene diamine
TRIS	: Tris (hydroxy methyl) amino methane
UV	: ultraviolet

Index

Part I

Fractionation, isolation and characterization

of **P**lacental **E**pap-1 **S**ecreting **E**ndothelial cells

1. Introduction

Pregnancy is a successful event of fertilization of ovum and sperm. Such a process is highly programmed and structurally organized for implantation, placental formation and embryonic development. During pregnancy all the metabolic, immunological and cellular activities favoring the fetal survival and development is predominant (Gharib-Hamrouche *et al*., 1993). Hence, several abnormal conditions are established in woman during pregnancy in terms of immunological, regulatory activities and nutritional status. Such activities would provide necessary growth conditions and protection against maternal stresses, such as microbial infection.

1.1. Embryo:

After the conception (fertilization of ovum with sperm), the zygote starts dividing mitotically and form morula (a ball of 12-16 cells called blastomeres). The morula enters the uterus and develops into blastocyst with a central cavity (called blastocoele) surrounded by a monolayer of cells the trophoectoderm (Norwitz *et al*., 2001). The zona pellucida covering the embryo ruptures as the blastocyst either begins to grow or is lysed by uterine/embryonic proteases. The hatched blastocyst then implants into the uterus. The trophoblast cell lining of the blastocyst are the first to differentiate into invading cell type that secrete human chorionic gonadotrophin (hCG) to maintain progesterone secretion by corpus luteum, which in turn is required for maintaining the pregnancy. (**Figure 1**)

1.2. Endometrial invasion

Trophoblast invasion of the maternal uterus is a multi-step process involving attachment of the trophoblast cells to the components of the ECM, and its degradation and migration of cells to maternal decidua (Fisher *et al*., 1989). A cell is considered to be invasive by virtue of

its ability to secrete proteases capable of digesting its immediate environment, and human cytotrophoblast cells falls under this category (Fisher *et al.*, 1989; Kliman *et al.*, 1990; Fisher *et al.,* 1985; Bischof *et al.*, 1992 and 1995). Cytotrophoblast production and activation of metalloprotease -9, which digests extracellular matrix in endometrium for the trophoblasts to invade between the epithelial cells of endometrium, is maximum during the first-trimester of pregnancy, coinciding with maximal invasive behavior *in vivo* (Librach *et al.,* 1991; Cross *et al*., 1994). Studies *in vitro* show, human cytotrophoblast invades acellular, amniotic membranes (Yagel *et al.,* 1988) as well as reconstituted basement Matrigel membranes (Bischof *et al.*, 1992). Cytotrophoblastic stem cells are capable of division throughout gestation, while a subset of post mitotic cells serves to replenish an outer syncytiotrophoblast layer after membrane fusion.

In addition to this endometrial invasion, groups of cytotrophoblast cells migrate through the decidua, invade the walls of the spiral arterioles, and replace the endothelial lining (a process called **endovascular invasion**) and the invasion proceeds into the myometrial segments of these blood vessels (**interstitial invasion**) (Fisher *et al.*, 1989). The net result is the formation of the human hemochorial placenta, in which blood from the maternal circulation constantly bathes the fetal chorionic villi throughout pregnancy.

Inadequate trophoblast invasion, has been implicated in the pathophysiology of pre-eclampsia, which is the leading cause of maternal death in the industrialized world and which increases the perinatal mortality by a factor of five (Norwitz *et al.*,2001).

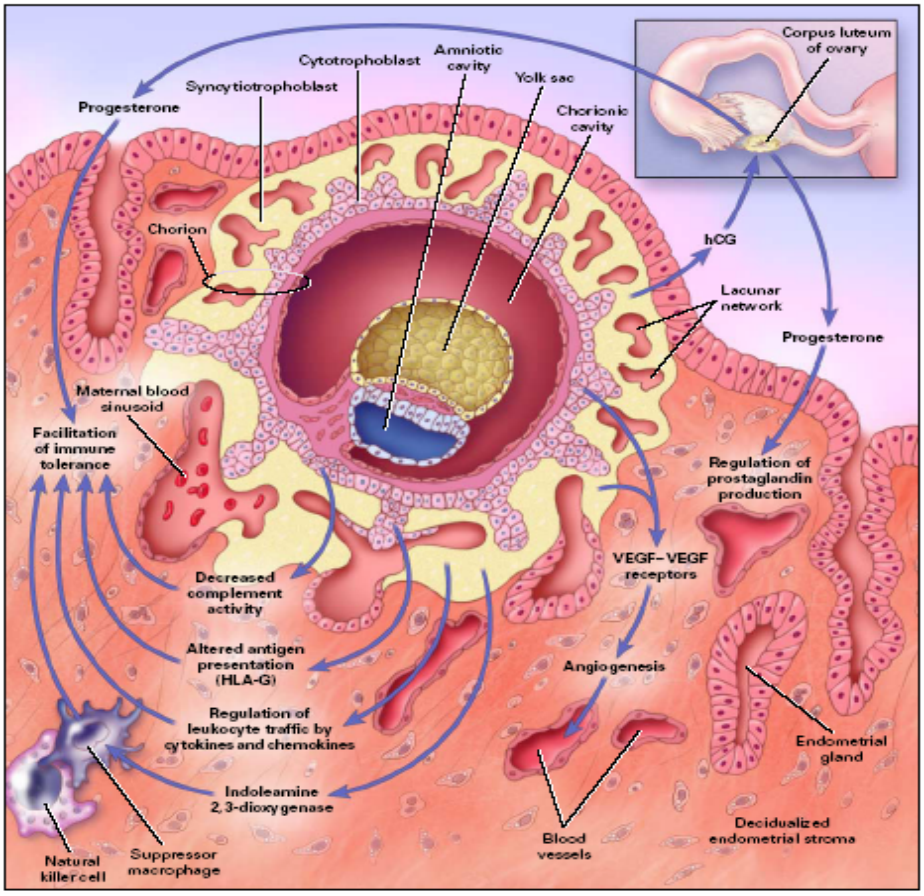


Figure 1.

Maintenance of Early Pregnancy.

The diagram shows an implanted embryo (approximately 14 days after conception) and the processes necessary for the maintenance of an early pregnancy. VEGF denotes vascular endothelial growth factor, and hCG human chorionic gonadotrophin. (Norwitz *et al.,* 2001)

1.3. Implantation:

The course of events during the implantation can be divided into three-stages:

In the first stage, the trophoblast cell lining present on the blastocyst undergoes morphological changes with decreased number of apical microvilli facilitating its closer association with the uterus. This initial adhesion of the cells of blastocyst to the uterine wall is called apposition. The next stage is a stable adhesion of cells, characterized by the inter-digitation of the micro-protrusions on the apical surface of the uterine epithelium, known as pinopodes. There is further increased physical interaction between the blastocyst and the uterine epithelium.

Shortly thereafter in the third stage, invasion begins, and syncytiotrophoblasts penetrate the uterine epithelium. The trophoblast cells exhibit highly invasive properties during implantation and they penetrate the uterine epithelium into the underlying stroma. As this penetration occurs, the trophoblast differentiates into two layers of cell types, the cytotrophoblast and the syncytiotrophoblast, which is a syncytium, made of maternal and trophoblastic nuclei.

Depending on their subsequent function in-vivo, undifferentiated cytotrophoblasts can develop into 1) hormonally active, **villous syncytiotrophoblasts** which are critical for maintaining pregnancy (Conley and Mason, 1990; Petraglia *et al*., 1990), 2) **extravillous anchoring trophoblastic** cell columns, promote attachment and are critical to the developing pregnancy. The cell to extracellular matrix interaction takes place at the attachment interface between the trophoblasts cells and the uterine wall. A specific type of protein fibronectin-trophouteronectin (TUN) is responsible for the attachment of anchoring, extravillous trophoblasts to the uterus throughout gestation (Feinberg *et al*., 1989, 1991). or 3) **invasive trophoblasts** attach to and interdigitate through the extracellular spaces of the endo- and myometrium (Kurman, 1991a,b; Kurman *et al*., 1984). This invasive behavior ends when the

invasive trophoblasts penetrate the maternal spiral arteries within the uterus to establish a materno-fetal circulation (Pijnenborg, 1981). Trophoblasts express various markers of extra cellular matrix (ECM) and proteases required for cell movement and invasion. In addition to these, trophoblasts also express a unique monomorphic histocompatibility antigen: HLA-G (Kovats *et al*., 1990) and provide immune tolerance to fetus.

1.4. Cells involved in placentation:

The establishment of a connection between the developing fetus and the mother's uterus is facilitated by the placentation during the early embryonic development in humans (Wilson, 2001). The major challenges for the newly fertilized embryo are to form a firm attachment with uterus and establish extensive surface area for nutrient/gases exchange (Gharib-Hamrouche *et al*., 1993). Various types of cells are involved in endometrial invasion, implantation and placental formation. These cells are briefly explained.

1.4.1. Maternal part of the placenta.

The fibroblast-like stromal cells of the edematous endometrium swell with the accumulation of glycogen and lipid droplets. They are called as **decidual cells**. The decidual cells form a tightly adherent, massive cellular matrix that first surrounds the implanting embryo and later occupies most of the endometrium. This development is called the decidua reaction. A primary function of the decidua reaction may be to provide an immunologically privileged site for the embryo. The decidua is divided into three regions, **decidua basalis** region is deep to the conceptus and forms the maternal part of the placenta, **decidua capsularis**, covers the conceptus and decidua parietalis denotes the rest of the endometrium. Many decidual cells degenerate near the embryo in the region of the syncytiotrophoblast. Together with maternal blood and uterine secretions, the decidual cells provide a rich source of nutrition. Another

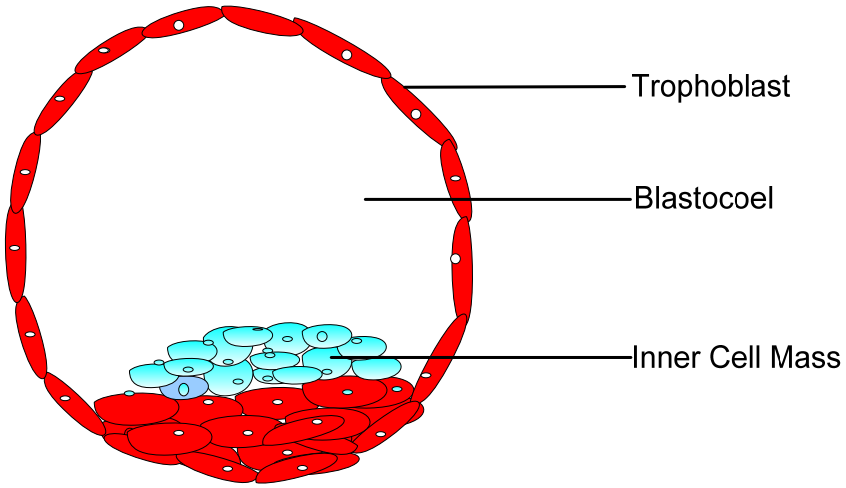
function of the decidua reaction may be to protect maternal tissue from uncontrolled invasion of the syncytiotrophoblast. Decidua cells may also be involved in hormone production.

1.4.2. The fetal part of the placenta.

The morula forms blastocyst with a fluid filled cavity, blastocoel. The blastocyst consists of an outer layer of trophoblast and an inner cell mass. (**Figure 2**)

B**lastocyst**

FIGURE 2



The trophoblasts layer differentiates into an outer syncytiotrophoblasts and an inner cytotrophoblasts. These cytotrophoblast cells send cords of cells into syncytiotrophoblast forming the primary chorionic villi. When mesoderm innervates the primary villi, secondary villi are formed which cover the entire chorionic sac. When mesenchymal cells differentiate into blood vessels, tertiary villi are formed.

Mesenchymal cells called angioblasts aggregate to form blood islands. Small cavities appear in the blood islands. Some angioblasts flatten to become endothelial cells and arrange

themselves around the cavities. Blood develops from endothelial cells. Vessels bud and fuse with other vessels.

The lacunae appear in syncytiotrophoblasts which are filled with glandular secretions and maternal blood. The maternal sinusoids (capillaries) anastomose with these lacunae to form uteroplacental circulation.

1.4.3. Circulation in the placenta

Maternal blood is discharged in a pulsatile fashion into the intervillous space by 80 to 100 spiral arteries in the decidua basalis. There is about 150 ml of maternal blood in the intervillous spaces, which is exchanged 3 or 4 times a minute.

During the first 12 weeks, the fluid in the intervillous spaces is a filtrate of maternal plasma without blood cells. During this period, the fetus has embryonic hemoglobin which binds oxygen under very low tension. After 12 weeks, maternal blood cells appear in the intervillous spaces, and the fetus produces fetal hemoglobin which requires a higher oxygen tension.

1.5. Placenta:

The placenta is the highly specialized organ of pregnancy that supports the normal growth and development of the fetus. Growth and function of the placenta are precisely regulated and coordinated to ensure efficient exchange of nutrients and waste products between the maternal and fetal circulatory systems. The main functional units of the placenta are the chorionic villi within which fetal blood is separated by only three to four cell layers (chorionic membrane) from maternal blood in the surrounding intervillous space. The

implantation of embryo into uterine wall endometrium is initiated by the outer layer trophoblast cells which proliferate and differentiate along two pathways described as villous and extravillous (Rockwell *et al.*, 2003). Non-migratory, villous cytotrophoblast cells fuse to form the multinucleated syncytiotrophoblast, which forms the outer epithelial layer of the chorionic villi. And at the terminal branches of the chorionic villi, majority of fetal/maternal exchange occurs.

Extravillous trophoblast cells migrate into the decidua and remodel uterine arteries. Migration and invasion of extravillous cytotrophoblasts into the maternal uterine epithelium are processes that are essential for increased uteroplacental blood flow as pregnancy progresses (Rockwell *et al*., 2003). During the modification and remodeling of spiral arteries, the muscular and elastic walls of the arteries are replaced with a fibrinoid layer embedded with trophoblast cells, allowing low-pressure intervillous flow (Kliman, 2000).

The syncytiotrophoblast cell layer, which is differentiated from cytotrophoblast cells, is the site where hormones such as estrogen, progesterone, hCG, placental lactogen, and placental GH are produced to maintain the pregnancy (Gluckman *et al*., 2002). Increased blood flow during pregnancy enhances the flow of nutrients from the mother to the fetus. The trophoblast invasion and changes in uteroplacental and umbilical blood flow are important for maintaining appropriate fetal growth through the supply of oxygen and nutrients.

The placenta continues to grow and develop throughout pregnancy and at term it is an organ of about 20 cm in diameter, with a thickness of 3cm and a weight of about 500 g. The chorionic villi have an enormous area of exchange, reaching 14 m^2 at term and the capillaries within them have a length of 50 km. The rate of maternal blood flow to the uterus is in the range of 500-700 ml/min with 80% of that delivered to the placenta (Gharib-Hamrouche et al., 1993).

1.6. Changes in surface receptor expression during endometrial invasion:

The specialized fetal cytotrophoblast cells differentiate and invade the maternal uterus to establish fetal attachment facilitating nutrient supply for the developing embryo. The cytotrophoblasts cells, the adhesive phenotype (fetal compartment) transforms to invasive phenotype replacing the maternal spiral artery in the uterus by changing its surface receptors. The cytotrophoblasts are of adhesive type in the embryo expressing extracellular matrix (ECM) proteins αVβ6 integrin and E-cadherin. These cells differentiate into invading phenotype as they invade the mother's uterus during the process the cells switch their receptors expression from adherent to invasive expressing vascular cell adhesion molecule (VCAM)-1, platelet-endothelial cell adhesion molecule (PECAM)-1 and integrins αVβ3 ,α1β. The invading cytotrophoblasts replace the vascular endothelial lining (Fisher *et al.*, 1989) of the spiral artery to form circulatory system. The significance of the replacement of endothelial cells with cytotrophoblasts expressing HLA-G is to avoid infiltration of immune competent cells into placenta and protect the allogenic foreign fetus.

1.7. Functions of the placenta:

The placenta is a metabolically active organ, the nutrients and metabolites are transferred across the placenta to the fetus by passive diffusion, facilitated diffusion, active transport, endocytosis, or exocytosis (Economides *et al.*, 1989; Sibley *et al.*, 1997). Transport of oxygen, carbon dioxide, and urea by passive diffusion is limited by the placental exchange area and blood flow. Facilitated diffusion of glucose and lactate involves transfer through a concentration gradient, which is mediated by a carrier molecule. Where as the active transport of other metabolites dependent on the energy as well as carrier proteins (Bauer *et al.*, 1998). The rate of nutrient transfer is directly proportional to that of fetal growth rate

(Sibley *et al.*, 2002). Further the exchange of metabolities also depend on the concentrations of substances in the lacunar network.

During early gestation, the primary function of placenta is to mediate implantation of the embryo into the uterus. Secondly, the placenta produces hormones that induce maternal recognition of pregnancy. After implantation, the major function of placenta is to mediate and regulate nutrient uptake from the mother to the fetus. This is accomplished by the formation of a highly branched villous structure and establishment of a circulatory connection with the umbilical cord.

1.8. Disorders of placentation:

Pre-eclampsia is a disorder specifically of human reproduction that manifests during the second half of 5–10% of pregnancies (Walker, 2000). It can be caused by incomplete remodeling of the spiral arteries, which leads to high maternal blood pressure and elevated concentration of urinary protein. Although originating in the placenta, the effects of pre-eclampsia become systemic: perturbation of maternal circulation and poor fetal growth (Redman., 2003). In the absence of care or intervention, pre-eclampsia can deteriorate into eclampsia, which threatens vital maternal organs and can lead to death for both mother and child. If only one of them dies, it is usually the mother. Worldwide, most pregnancy-associated mortality is due to pre-eclampsia and eclampsia. Convulsions typify eclampsia, hence the name, which is derived from the Greek word, "*eklampsis*", for a sudden flash or development (Fisher, 2004**)**. The only known cure for pre-eclampsia is to deliver the fetus, the physician's challenge being to balance the needs of both mother and child in deciding when to intervene with either drug-induced labor or a cesaerian section (Walker, 2000). Hearsay and clinical observation pointed to the possibility that polymorphisms in both maternal and paternal genes contribute to pre-eclampsia. Maternally, pre-eclampsia runs in

families, and some men seem prone to fathering pre-eclamptic pregnancy (Moffett-King, 2002.). In this issue, Hiby *et al*. implicate that HLA-C on fetal trophoblast cells and KIR on uterine NK cells as factors affecting pre-eclampsia (Hiby *et al.*,2004).

Abnormalities in the invasive process cause early and mid-trimester pregnancy loss, pre-eclampsia and eclampsia, and intrauterine growth retardation (Pijnenborg *et al*., 1981). It appears in two stages: the preclinical phase is characterized by failure of cytotrophoblast invasion of maternal spiral arterioles, leading to a hypoxic placenta and, hence, upregulation of the production of hypoxic-responsive placental factors secreted by the trophoblast, including components of the VEGF/PlGF pathway. In turn, this local defect leads to the second phase of pre-eclampsia, whereby systemic responses in the mother result in hypertension, proteinuria, blood clotting and various other internal organ dysfunctions predominantly due to endothelial dysfunction. Pre-eclampsia can be life threatening to both mother and baby and can be cured only by correcting delivery of the placenta. Recent evidence has suggested that increased circulating levels of sFlt1, along with reduced levels of VEGF and placental growth factor (PIGF) in maternal serum 5 weeks before clinical onset of pre-eclampsia, can be predictive factor of disease (Levine *et al*., 2004). Importantly, delivery of sFlt1 directly to pregnant rats recapitulated many hallmarks of pre-eclampsia, including hypertension, proteinuria and kidney endotheliosis (Maynard, *et al.,* 2003). Therefore, blocking sFlt-1 is an attractive avenue to manage pre-eclampsia. The finding that Flt1 is not required for placental development itself in mice is encouraging, as it suggests that there might not be unwanted placental side effects of any such therapy.

1.9. Production and regulation of hormones.

Placenta plays an active role in regulating maternal physiology towards the nutritional benefits for the fetus. This is achieved through angiogenic factors and vasodilators produced by trophoblasts that invade the arteries causing increased blood flow to the implantation site (Cross *et al.,* 2002 a,b). In this "autocrine regulatory model", the hormones produced in the placenta can stimulate and increase the production of maternal blood cell population and blood volume (*Conrad et al., 1996; Kim et al., 2001; Zhou et.al.,2001, 2003, 2005).* Placental lactogen and placental growth hormones alter insulin production and promote insulin resistance in maternal tissues favoring increased glucose availability to the fetus (Linzer and Fisher, 1999). The placenta also produces leptin (Pelleymounter *et al.,* 1995a,b) and Ghrelin (Tschop *et al.,* 2000), hormones that suppress and stimulate the appetite respectively. Synthesis of hCG begins before implantation, and is responsible for maintaining the maternal corpus luteum that secretes progesterone and estrogens (Ohlsson *et al.*, 1989). The placenta can produce progesterone independently from cholesterol precursors, and estrogen in concert with the fetal adrenal gland, as it does not contain all the necessary enzymes itself (Nulsen *et al*., 1989). Human placental lactogen (hPL) is synthesized throughtout gestation, increasing progressively until the 36th week (Sakbun *et al*., 1990a). Prolactin (Al and Fox, 1986 and Sakbun *et al*., 1987), relaxin (Sakbun *et al*., 1990b) and chorionic adrenocorticotropin (Saijonmaa *et al*., 1988, Odagiri et al., 1979; Krieger, 1982; Liotta et al., 1977) are synthesized by placental cells that function similar to pituitary hormones. It appears from these studies that the placenta, in addition to replacing much of the women's pituitary function during pregnancy, so as to maintain control and feedback loop mechanisms close to the conceptus.

1.10. Maternal immune regulation during fetal development:

The trophoblastic epithelium of the placenta maintains a high concentration of steroid and protein hormones that are believed to confer immuno-protection to the growing fetus. The increased levels of progesterone at the placental/ decidual border are equivalent to the in vitro levels, which inhibit lymphocyte responses to mitogens or allogeneic cells. This hormone may thus play a significant role in neutralizing maternal immune responses against the fetus (Clemens, *et al*., 1979). Similarly, the activity of lymphocytes can be suppressed in vitro by a variety of substances synthesized in the course of a normal pregnancy including, human Chorionic Gonadotropin (hCG), human Placental Lactogen (hPL), prolactin, cortisone, estrogens and a host of other proteins and glycoproteins (Lawrence, *et al*., 1980, Peyman, *et al.,* 2001).

The uterus also produces high concentrations of Transforming Growth Factor (TGF β) and prostaglandin E2 (PGE2), both are potent immunosuppressive agents (Das. *et al*., 1992, Wood, 1994). Placental production of anti-inflammatory cytokines, TGF β2, Interleukin (IL)-4 and IL-10 may counteract the deleterious effects of inflammatory cytokines IL-2, Interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α on the fetus (Moulton, 1993, Chaouat, 1995).

Interferon β produced by the human trophoblast, is reported to have marked immunosuppressive effects on the mitogen-induced proliferation of human T-cells and B-cells *in vitro* in a dose-dependent manner, suggesting that the local cytokine network at the feto-maternal junction may also play an important role in the immunobiology of human pregnancies. Uterine NK cells express the highest amounts of CD56 and are also skewed toward cytokine secretion (Cooper *et al*.,2001; King *et al.*, 1991).

Trophoblast cells also abundantly express Fas-L (or CD95- L). Fas-L expression has been proposed to contribute to immune privilege in this unique environment, by fostering apoptosis of activated Fas-expressing lymphocytes of maternal origin (Uckan *et al*., 1997). hCG may be a crucial link in the development of peritrophoblastic maternal immune tolerance and may facilitate trophoblast invasion by regulating the Fas-Fas-L system (Kayisli *et al*., 2003).

1.11. Allogenic fetus immune tolerance:

Since the fetus contains genetic material from both the mother and the father it is said to be semi-allogenic for the mother, this means that the mother and fetus would have different major histocompatability complexes (MHC). Thus fetus would be rejected in a fairly short time as in a transplantation or graft, it would also mean that any future fetus with the same father would be even more quickly rejected by the mother. There evidence to suggest that having different MHC's is beneficial (Roitt, 1991)

In humans, the mothers blood (with immuno competent lymphocytes) is in direct contact with fetal trophoblast. Hence, there exists some mechanisms to protect the fetus from rejection. The most important factor is the lack of both class I and class II MHC antigens on the chorionic villi, thus giving the fetus protection against allogenic attack by the mothers immune system (Wegmann *et al.,* 1979). It has been found that some populations of cytotrophoblast cells produce a unique class I MHC molecule that has been named HLA G. HLA G has some interesting properties that may mean that it is an integral part of the defence mechanism of the feto-placental unit, for example HLA G transfected L cells are more resistant than control ones to natural killer cells or T cell mediated lysis (Chaouat and Menu, 1994).

Cytokines seem to be important in post-implantation pregnancy given that both granulocyte macrophage-colony stimulating factor (GM-CSF) and transforming growth factor-ß (TGF-ß) are produced by the maternal endometrium (Roitt, 1991)

1.12. HIV and pregnancy:

In pregnancy due to hormonal changes or due to the suppression of cellular immunity there is an accelerated decline of immune function associated with human immunodeficiency virus (HIV) infection (Tuomala *et.al*., 1997). Thorne *et al,* (1995) estimated that 66% of the women with a CD4 count of less than 200 cells/mm^3 will progress to stage 4 within a period of 2 years after delivery, in contrast to only 21% of women with a CD4 count of more than 499 cells/mm3. Whether HIV-1 infection itself may adversely affect pregnancy remains controversial. Brocklehurst & French (1998) observed an association between the risk of spontaneous abortion and women infected with HIV-1 that varied from 1.8 to 6 times greater. HIV-infected patients often exhibit hypergammaglobulinemia, a potential cause of false-positive results through nonspecific binding of IgG (Maclean, 1990). Adverse pregnancy outcome has frequently been reported in HIV infection (Brocklehurst & French, 1998). There may be a direct effect of HIV- 1 on the placenta and on embryogenesis. Immunosuppression caused by HIV infection, facilitating the ascension of infections from the lower genital tract and the development of viral or bacterial infection of the villi (D'ubaldo, 1998) are some of the frequently associated complications in HIV-1 infected pregnant women. (Elizabeth et al., 2003, **Figure 3**).

1.13. Vertical transmission of HIV-1:

The vertical (mother-to-infant) transmission of human immunodeficiency virus type 1 (HIV-1) occurs at an estimated rate of 10-39% (Domachowske, 1996; Zachar *et al., 1999)* and is the major cause of AIDS in children. Numerous maternal parameters, including advanced clinical stages, low CD4+ lymphocyte counts, high viral load, immune response, and disease progression have been implicated in an increased risk of vertical transmission (Ahmad, 2005) HIV can be transmitted from mother-to-child at various stages of pregnancy including in utero, intrapartum and during breastfeeding. DC-SIGN was expressed, cloned from human placenta in 1992 because of its ability to bind the HIV envelope protein gp120 (Curtis *et.al*., 1992; Geijtenbeek *et al*.,2000). In 2000, Geijtenbeek *et al*. studied on the dendritic (DC)-expressed non-integrin ligand for ICAM-3. DC-SIGN-positive DCs incubated with HIV are able to infect T lymphocytes very efficiently, even after very thorough washing. This process is referred to as potentiation of HIV infection in trans (Geijtenbeek *et al*., 2000). DC-SIGN expression occurs on two cell populations in the placenta, namely maternal decidual macrophages and fetal Hofbauer cells in the connective tissue core of chorionic villi (Soilleux *et al*., 2001).

Worldwide every year approximately 7,50,000 children become infected with HIV, mostly through mother-to-child transmission (MTCT). Without specific interventions, the rate of MTCT is approximately 15-20%, with prolonged breastfeeding doubling the rate to 35-40%. Current approaches of intervention to reduce the risk of MTCT focus mainly on antiretroviral prophylaxis during pregnancy, labour and in the early neonatal period, but in some settings also on delivery procedures and avoidance of breastfeeding (Newell, 2005). Large-cohort studies in North America, Europe, and Thailand have shown that azidothymidine (AZT) monotherapy, given at the late stages of pregnancy, is of proven

benefit in reducing mother-to-infant HIV transmission by 51% to 68%. Emergence of AZT-resistant variants in pregnant mothers (7-29%) and their infected offspring (5-21%) has been described in several studies. Drug resistance arises more frequently in those mothers who received AZT therapy before pregnancy. Recent advances in combination chemotherapy may provide alternative strategies in prevention of vertical transmission and drug resistance (Brenner, 2000; Newell, 1998).

1.14. Anti-viral barriers in placenta

Placental development is directed towards the establishment of a continuous nutrient supply to the developing fetus. This requires efficient access of maternal blood to a transporting surface, the multinucleate syncytiotrophoblast layer (Newell, 1998). Maternal-infant transmission of human immunodeficiency virus type-1 (HIV-1) is the primary cause of this retrovirus infection in neonates. Hofbauer cells, specialized fetal macrophages, are known to be involved in HIV infection *in vitro* and cell infection *in vivo* has been demonstrated by hybridization, PCR *in situ* and immunohistochemistry (Soundararajan, 2004). The major barrier between Hofbauer cells and maternal fluids is the trophoblast layer. Although there are reports that this may become infected with HIV (Sheikh, 2000), trophoblast cells are reported to be only "moderately susceptible" to HIV infection *in vitro* (Moussa *et al*., 1999). Placentae from HIV-infected women treated with HAART, who gave birth to uninfected babies showed no evidence of HIV infection of trophoblast (Tscherning-Casper *et al*., 1999). Hence, placenta possesses an efficient barrier in preventing the HIV-1 transmission. Such barrier may involve certain cell populations that express various antiviral factors (Table 1) present in circulation during early and late pregnancy. These observations unequivocally demonstrate the presence of innate anti-viral environment in the placenta that

controls the viral load to prevent viral transmission to the fetus. It would be important to understand the cellular mechanisms associated in control of viral transmission in placenta.

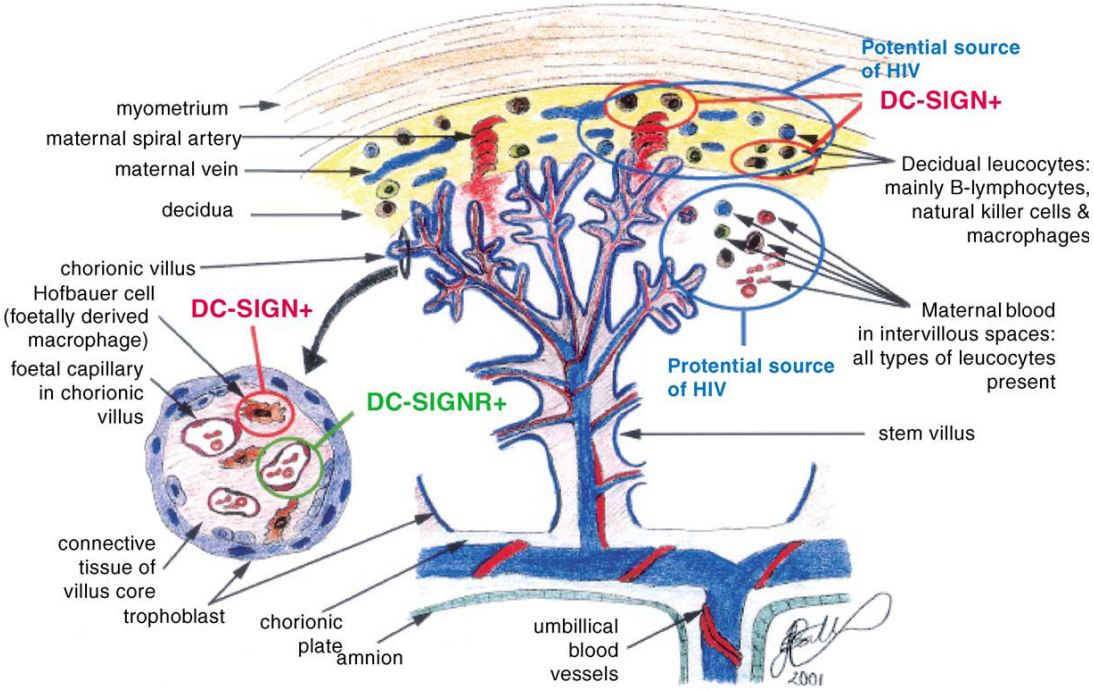
1.15. Rationale

Our laboratory has identified and characterized an innate immune factor, Epap-1 in the first trimester placental tissue. (Kondapi *et al., 2002)*. In the present study we have investigated various cell population in placental tissue that are associated with Epap-1 expression with an aim to understand the cellular mechanisms of Epap-1 mediated protection from HIV-1. Such an information can help in the development of future intervention in regulation of Epap-1 mediated anti-HIV-1 environment during pregnancy.

Objectives:

1. Isolation of Placental Epap-1 Secreting cells.
2. Characterization of isolated Placental cells.
3. Purification and characterization of Epap-1 from spent medium of Epap-1 secreting placental cells
4. Analysis of anti-viral properties of placental cells.

FIGURE 3



Simplified Structure of Human Placenta

TABLE 1

The molecules that regulate the immunity at feto-maternal interphase and protect from pathogens are listed below

Anti-viral factor	Produced by	References
Anti-HIV-1 gp120/gp160 & P17/p18 antibodies	Extra villous trophobalsts (EVT) (Second & third Trimester)	Lyden *et al.,* (1995) J. Reprod. Immunol. 28(3), 233-45
Placental factor(~55 KDa)	Placental stromal cells	Sharma *et al.,* (1998) J. Immunol. 161, 6406-12
γd & αδ T cells (mid gestation)	Anti-viral autocrine and paracrine factors	Hunt *et al.,* (2000) Semin. Cell. Dev Biol. 11(2), 127-37
Collectins (c-type lectin)	Membrane of placenta	Ohtani *et al.,* (2001) JBC 276 (47), 44222.
Leukemia Inhibitory Factor IFNγ	Humoral and cellular immune resposes of placenta	Patterson *el al*., (2002) Curr. Mol. Med. 2(8), 713-22
Killer specific secretory protein (37 KDa, Ksp37)	Expressed by CD16+ NK cells(in decidua) (late pregnancy)	Hayano *et al*., (2002) Am. J. Reprod. Immunol. 48(1) 57-62.
Epap-1(90 KDa)	**First trimester placental tissue**	**Kondapi *et al*., (2002) Antiviral Res. 54(1), 47-57.**
Indoleamine-2,3-deoxygenase (IDO) (Absent in 1st Trimeter	Present at endomatrial grandular & surface epithelial cells.	Sedlmyr (2002) Mol. Hum. Reprod. 8(4), 385-91.

2. Methodology

Materials

The various biochemical's used in the study were purchased from the following companies or obtained as gifts from other laboratories as mentioned below: AZT, 3'-azido-3'-deoxythymidine, Calcein AM and Calcein blue AM (Molecular probes), pG105-T easy vector kit was from Genotix ; Qiaprep Spin Miniprep kit were from Qiagen. Mono S ion exchange matrix was from Amersham Biosciences, *Sambucus nigra* Agarose lectin was procured form EY Labs, U.S.A. All the p24 antigen capture assays were done using **HIV-1 p24 Antigen Capture Assay Kit** (NCI-Frederick Cancer Research and Development Center). All other chemicals were from Sigma (St. Louis, MO) unless specified. Polyclonal antibody against Epap-1was raised in rabbit by my senior, Dr. M.A. Hafiz, in our laboratory.

Cells Lines, Vectors and Antibodies:

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA. NIH 3T3 bearing CD4 and CXCR4 or CCR5(Dr. Deng); Chinese hamster ovary (CHO) JRL, clone A 9; HL 2/3 cells (Ciminale,1990) expressing IIIB HIV-1 Env were cultured in Dulbecco's modified Eagle medium (DMEM-F12) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). SupT1 cells were cultured in Roswell Park Memorial Institute medium 1640 (RPMI 1640) (Invitrogen, Carlsbad, CA) with 10% FBS. The various epitope specific gp120 Monoclonal antibodies V2 specific: 697-30D(Gorny MK, 1994), V3 epitope: 257-DIV (cf 257D) and C5 region: 670-30D (Dr. S. Zolla-Pazner); V3 loop: V3-21, SVEINCTRPNNNTRKSI, 298-315 (Dr. J. Laman); V3 loop: F425 B 4a.1 (cf F425) and gp41: F240 (Dr. M. Posner and Dr. L. Cavacini); CD4 Mab: SIM4 (Dr. J. E. K Hildreth); C3 reactive: B32-FFY, 382-384 (Dr. G. W.Lewis).

The HIV-1 isolates used in the study are as follows The following two HIV-1 strains were procured form NIH-AIDS Research and reference reagent program, USA:

HIV-$1_{93IN101}$: is of biotype-NSI (X5) (R.Bollinger) isolated from a seropositive individual in India. Procured form NIH-AIDS Research and reference reagent program, USA. Contributor

HIV-1_{MN}: is of biotype-SI (X4) (Gallo, 1984). This strain exhibits the same cytopathic effects on H9 cells as HTLV-1-IIIB for DAIDS, NIAIDS and the UNAIDS Network for HIV isolation and characterization. Procured form NIH-AIDS Research and reference reagent program, USA

HIV-1_{CEM-50}: was obtained form Dr. Robin Mukhopadhyaya, Cancer Research Institute Tata Memorial centre. Mumbai, India.

HIV-1_{HYD1}: is a local strain isolated by authors from a HIV-1 seropositive 18 year old pregnant woman from Hyderabad, India and propagated in SupT1 celline and PBMCs. The virus replicated in the SupT1 cells was used for the study.

2.1. Isolation of PBMCs from human blood:

10 ml of blood was collected form healthy volunteers and diluted it to 1:1 ratio with saline. Diluted 20ml blood was layered on 10 ml of histopaque in a 50 ml round bottomed centrifuge tube. The sample was subjected to centrifugation at 400 x g for 20 minutes in a bucket centrifuge (Heareus) without applying brakes. The clear white band of cells is seen at the histopaque gradient. The cells were carefully pipetted out without disturbing the contents in the tube and resuspended in saline (50ml). This was again centrifuged at 1200 rpm for 10 minutes. The supernatant is discarded and the pellet was washed 3 times in saline. The cells were seeded at density of 1x 10^6 cells /ml in RPMI 1640 containing 10% FCS and 2µg of PHA. The cells were cultured at 37^0C and 5% CO_2.

2.2. Isolation of Trophoblast cells form placental tissue

The MTP placental tissue was washed under sterile condition with 200ml of PBS, pH 7.3 until it is free of blood. The tissue was minced, the cells were disaggregated and three sequential 10 minutes treatments with 0.125 % trypsin and 0.2 mg/ml of DNase was done (George et al., 1996). The cells released at each treatment were filtered through 2 layers of muslin cloth. At the end of the treatments 10% of serum was added to inactivate the action of trypsin. Cells were resuspended in 70% percoll at a density of 2 x 10^5 cells per ml and the mixture was layered under 20 ml of 25% percoll and 10 ml of PBS was put on top of the 25% percoll. The sample was subjected to a spin at 2000rpm in a bucket centrifuge (Heraeus) with out applying brakes for 20 minutes. After the spin, the middle band at density of 1.048 to 1.062 g/ml was collected gently without disturbing the other contents of the sample. It was then washed with 50ml PBS. The cells were seeded in 10ml of DMEM with 10%FCS, at a density of 1 x 10^6 / T 75^2 tissue culture flask and incubated at 37^0C with 5% CO_2.

2.3. SDS-PAGE analysis:

Polyacrylamide gel electrophoresis was performed according to the method of Lammelli (1970) in 0.1% SDS on a vertical slab gel system. The gels contained 10% acrylamide with 30:1 ratio of acrylamide to N, N, N', N'- methylene-bis-acrylamide. Samples were boiled at 100^{o}C for 10 min in the presence of sample buffer to dissociate proteins into their individual polypeptide chains. The loading dye contained: SDS (1% w/v), 2-mercaptoethanol (5% v/v) in 0.063 M Tris.HCl, pH 6.8. The protein mixture was subjected to electrophoresis on 4.5% (w/v) Polyacrylamide stacking gel in 0.125 M Tris-HCl, pH 6.8 and 10% (w/v) resolved gel in running buffer 0.375M Tris. HCl, pH 8.8 at 100v till the dye front reaches the end of the gel. The electrode buffer (pH 8.5) contained 0.025M Tris buffer, 0.192M glycine and 0.1% SDS.

2.4. Silver staining :

Silver staining of the proteins was done as described by Blum *et.al.,*(1987). Briefly, gels were fixed in fixative solution (50% methanol, 12.5% acetic acid and 0.75% of 37% formaldehyde) for 1 hour followed by washing thrice with 50% alcohol for 30 min each. Gels were treated with sodium thiosulphate (0.2g /liter) precisely for 1 minute and then washed with double distilled water (DDW) to remove excess of thiosulphate. The gels were treated with freshly prepared silver nitrate. (2g/ litre of DDW containing 0.5% of 37% formaldehyde/ liter) for 30 minutes on a shaker. Excess of silver nitrate was removed by washing thrice with DDW. And finally the gel was developed using 6% sodium carbonate containing 0.75% of 37% formaldehyde/liter). As soon as the protein bands of required intensity appeared, the reaction was stopped by the addition of 7.5% acetic acid and the gels were preserved in 50% ethanol.

2.5. Western Blot analysis:

The samples electrophorised on 10% gel was transferred onto a nitrocellulose membrane. Protein transfer was performed at 45 mA for two and half-hour in towbin buffer (25mM Tris-HCl (pH 8.3), 192 mM glycine, 20 % methanol) in a Bio-Rad western-blot apparatus (Towbin *et al.,* 1979). Protein transfer was checked with Ponceau-S, a reversible protein stain. After blotting the membrane was washed with TBS (10mM Tris pH 8.0, 150mM sodium chloride) and non-specific binding sites were blocked with 5% non-fat dry milk powder in TBS for 1h at room temperature. The blots were then probed with Rabbit anti-human Epap-1 polyclonal antibody for 1h at room temperature. The membranes were washed thrice with TBS (TBS containing 5% Tween-20) to remove excess of the primary antibody and incubated with goat anti-rabbit IgG (H+L) conjugated to Alkaline phosphatase in TBS buffer. The membrane was then washed thoroughly in TBST thrice to remove unbound antibody. Color was developed in ALP buffer (10mM Tris, 5mM $MgCl_2$ and 100mM NaCl, pH 9.5) containing 0.033% NBT and 0.0165 % BCIP. The reaction was stopped by washing the membrane with distilled water. The membrane was dried and densitometric analysis was performed using a color scanner (HP 5100C) and the NIH Image software (Scion Image).

2.6. Isolation of Placental cells from MTP placental tissue.

The MTP (Maternally terminated Pregnancy) placental tissue was washed in sterile condition with 200ml of PBS, pH 7.3 to remove blood and collect placental villous tissue. The tissue was minced and incubated with Trypsin (0.12%) and DNase-I (5mg/10ml) in 10ml DMEM at 37^0C for 10-15 min. The trypsin was inactivated by addition of 1ml of FCS (10%). The treated tissue was passed through 40 micron nylon mesh to remove large undigested tissue. The filtrate was centrifuged for 1000 rpm/10min. The cell pellet was resuspended in

1ml DMEM and layered on 5-70% Percoll gradient in saline. The gradient was centrifuged at 400g (approx 1400 rpm) in a bucket centrifuge (Heareus) for 20 minutes without brakes. The cells separated at the center of the tube with a density of 1.087 were taken and washed with 25ml of 1X PBS. The cells were seeded in 10ml of DMEM with 10%FCS, at a density of 6 x 10^6 / T 75^2 tissue culture flask and incubated at 37^0C with 5% CO_2. After 24 hours, the adherent and non adherent cell populations were separated and seeded into separate flasks. In one set from each set of adherent and suspension cultures of flasks, mitotic inhibitor 5-Fluoro-deoxy Uridine (FDU) was added and cells were cultured.

2.7. Purification of Epap-1 from placental culture supernatants (Spent medium):

2.7.1. Ammonium Sulphate precipitation: The proteins in PESE cell culture supernatants were fractionated with ammonium sulfate at 0-60%, saturation. The pellet was discarded and proteins present in the supernatant were refractionated with ammonium sulphate at 80% saturation. 60-80% fractionated proteins were dissolved in minimum volume of PBS and dialyzed extensively against PBS pH 7.3 overnight at 4^0C. The dialyzed fraction was used for further purification and binding studies. The dialysate was centrifuged at 8000 x g for 10 min at 4^0C. The proteins in the supernatant were estimated using Bradford colorimetric method (Bradford, 1976)

2.7.2. Epap-1 purification from placental cell culture supernatants (spent medium) by *Sambucus nigra* Affinity chromatography: 10mg of total protein from 60-80% ammonium sulphate precipitated fraction was loaded onto 5ml immobilized *Sambucus nigra* agglutinin lectin-agarose (Broekaet *et.al., 1984)* affinity column. Flow through was collected and the column was washed with 50 bed volumes of PBS until OD_{280} of the flow through reaches below 0.01. The protein was eluted with PBS, pH7.3 containing 50mM D (+) galactose in 1 ml fractions. The eluted protein was dialyzed extensively and analyzed immediately using 10% SDS-PAGE followed by silver staining and Western blotting. High molecular weight protein standard marker was used.

2.7.3. Mono S cation exchange chromatography: Dialysate of **60**-80% ammonium sulphate precipitate protein was loaded onto 2 ml immobilized Mono S matrix pre-equilibrated with 50mM phosphate pH 7.2. Flow through collected and washed with 50mM phosphate pH 7.2. The bound protein was eluted with 50 and 100mM KCl in 50mM phosphate buffer. The protein elutes were dialyzed and analyzed on 10%SDS PAGE followed by silver staining.

2.7.4. Leupeptin column chromatography: The 60-80% protein dialysate (1 mg) was loaded onto 2ml of immobilized Leupeptin matrix, equilibrated with PBS pH 7.3. the flow through was collected and washed with 50 bed volumes of PBS pH 7.3. The LPP bound protein was eluted with 50mM galactose in phosphate buffer, pH 6.0. The elutes were dialyzed and analyzed on 10% SDS PAGE followed by silver staining.

2.7.5. Phenyl-Sepharose column chromatography: The 60-80% protein dialysate was loaded onto 2 ml of immobilized Phenyl-Sepharose matrix, equilibrated with PBS pH 7.3. The flow through was collected and washed with 50 bed volumes of PBS pH 7.3. The Phenyl

Sepharose bound protein was eluted with 20% isoproponal in PBS. The elutes were dialyzed and analyzed on 10%SDS PAGE followed by silver staining.

2.8 Selection for Pure culture:

The total cell types isolated from the percoll gradient (as mentioned earlier) were cultured and were selectively grown in presence of a mitotic inhibitor, 5-Fluro deoxy Uridine (FDU) at 100μM concentration for 3 days. The cells were serially diluted and cultured in DMEM F12 medium with 20% FCS. The cells were scored for Epap-1 secretion and were further cultured and characterized. Pure culture obtained was characterized with various cell type specific markers, morphology and activities.

2.9. Characterization of the pure culture with various cell type specific markers:

2.9.1. Growth properties: The pure cultures were phenotypically characterized using inverted and confocal microscope. The cells grow without requirement of any additional growth factors with a doubling time of 4 days. The cells grow as self-adherent mono-layer and multi-layered cells. Morphologically the cells are transparent with extended cell structure containing a single nucleus. They show the formation of surface filaments. The cell colonies are in propagation for the past 24 months.

2.9.2. Immunofluorescence The selected PESE cells were seeded in 12 well dishes on cover slips (poly-D-lysine coated). The cells were cultured for 3 days at 37^0C with 5% CO_2. the cells were washed with saline gently and fixed on to the slides with 3% paraformaldehyde in PBS for 15 minutes, washed with wash buffer (0.1% Azide in PBS) thrice. Blocked with 3% BSA for 30 min for 1 hour. Washed twice with wash buffer. The fixed cells were incubated

with mouse anti-human cytokeratin, vimentin, CD9 monoclonal antibodies and rabbit anti-human Epap-1 polyclonal antibody for 1 hour at 37^0C. Slides were washed thrice and incubated with anti-mouse IgG-FITC for 1 hour at 37^0C (in dark). The slides were washed thrice with wash buffer and the fluorescent labeled cells were observed under Leica confocal microscopy.

2.9.3. FACS analysis: The placental cells were washed in DMEM F12 medium and suspended to $0.5x10^6$ cells/0.1ml. The cells were incubated at 1:500 dilution of mouse anti-human cytokeratin, vimentin, CD9, hCG monoclonal antibodies and rabbit anti-human Epap-1 polyclonal antibody at 4^0C for 1 hour. The cells were washed twice in DMEM F12 containing 0.02% azide. They were incubated with anti-mouse IgG-FITC (1:500 dilution) at 4^0C for 1 hour. The cells were washed thrice with DMEM F12 containing 0.02% azide. The cells were fixed with 1 % paraformaldehyde in PBS for 20 minutes, washed with PBS thrice. The cells were suspended in sheath fluid (1 ml) and analyzed by FACS.

2.10. PESE cell anti-viral assay:

$0.5x\ 10^6$/ml/well PESE cells were cultured in 12 well plate for 3 days at 37^0C and 5% CO_2. In one set, spent medium of PESE was removed and fresh complete medium (DMEM F12+ 10% FCS) was added. In second set, the spent medium was kept intact. Both sets of PESE cultures were challenged with 0.5ng/ml p24 equivalent of HIV-1 (strains HIV-$1_{93IN101}$, HIV-1_{CEM50}, HIV-1_{UH1} and HIV-1_{MN}). The control infection was conducted in two-day-old PHA stimulated $0.5x\ 10^6$/ml/well PBMC with 0.5ng HIV-$1_{93IN101}$. The cells were cultured for 4 days and the culture supernatants were quantified for the HIV-1 p24 antigen using P24 Antigen Capture Assay.

2.11. PESE cell anti-viral dynamics assay:

0.5x 10^6/ml/well PESE cells were cultured in 12 well plate for 3 days at 37^0C and 5% CO_2. In one set, PESE culture medium was removed and fresh complete medium (DMEM F12 + 10% FCS) was added. In second set, the 3 day spent medium was kept intact (which containing all the secretary products of the cell). These cells were further split into two groups. In first group, the cells were allowed to incubate for 1, 3, 6 and 12 hours, then they were challenged with 0.5 ng p24 equivalent of HIV-1 $_{93IN101}$. While in the second group, the virus was added and (cells with spent medium) the virus continuing medium was washed at 1, 3, 6 and 12 hours of postinfection. The washed cells were re cultured and the virus replicated for 4 days was quantified for the HIV-1 p24 antigen using p24 Antigen Capture Assay.

2.12. PESE cell-PBMC co-culture assay:

0.5 x 10^6 /ml/well PESC cells were seeded in 12 well plate at 37^0C and 5% CO_2 for 3 days. In one set, 3 day spent medium containing PESE cells were co-cultured with PBMCs (0.5x 10^6/ml/well, 0.2 μg/ml PHA stimulated). Second set, PESE cells without spent media were co-cultured with PBMCs as described below,

PESE cell+ 3 day spent medium + PBMCs

1. PESE cell + No spent medium + PBMCs
2. PESE cell + 3 day spent medium
3. PESE cell + No spent medium
4. PBMCs

The co-cultured cells were challenged with HIV-1 $_{93IN101}$, the amount of virus replicated at day 4 in the culture supernatants were quantified for the HIV-1 in terms of p24 antigen using P24 Antigen Capture Assay.

2.13. PESE cell-Trophoblast cell co-culture assay:

0.5 x 10^6 /ml/well PESC cells were seeded in 12 well plate at 37^0C and 5% CO_2 for 3 days. In one set, the spent medium was removed to the 3 day culture of PESE cells were co-cultured with Trophoblast cells (0.5x 10^6/ml/well). In second set, the spent medium was not removed and the PESE cells were co-cultured with trophoblast cells as described below,

1. PESE cell + 3 day spent medium + Trophoblast cells
2. PESE cell + No spent medium + Trophoblast cells
3. PESE cell + 3 day spent medium
4. PESE cell + No spent medium
5. Trophoblast cells

The co-cultured cells were challenged with HIV-$1_{93IN101}$, the amount of virus replicated at day 4 in the culture supernatants were quantified for the HIV-1 in terms ofp24 antigen using P24 Antigen Capture Assay.

2.14. PESE cells- chronically infected cells co-culture:

0.5x 10^6/ml/well PESE cells were seeded in 12 well plate for 3 days at 37^0C and 5% CO_2. PESE cells were incubated for 3 days. These cells were co-cultured with chronically infected SupT1 cells (with HIV-$1_{93IN101}$) with and without spent medium as follows

1. PESE cells + 3 day spent medium + Chronically infected SupT1 cells
2. PESE cells + No spent medium + Chronically infected SupT1 cells
3. PESE cells + 3 day spent medium
4. PESE cells + No spent medium
5. Chronically infected SupT1 cells

The amount of virus replicated at day 4 in culture supernatants were quantified for the HIV-1 p24 antigen using P24 Antigen Capture Assay.

3. Results and Discussion

HIV vertical transmission is highly variable. The rate of transmission to the fetus depends on the viral load of women during pregnancy (Coll *et al.,* 1997**).** It has been shown that the viral load of HIV seropositive women decrease upon conception and during pregnancy, indicating the activation of certain control pathways *in vivo* to provide antiviral environment in decreasing viral infectivity. The goal of present study is to identify one of the pathway involving, Early pregnancy associated protein-1 (Epap-1). Since our earlier studies showed that Epap-1 cDNA and protein are expressed in placental tissue, this tissue may be associated with certain cells that express Epap-1. The aim of present study is to identify the cells that can express Epap-1 and study their anti-viral properties *in vitro* to understand the mechanism of innate immunity mediated by Epap-1 and building a hypothetical model, a possible alternative model for natural protection of fetus form circulating virus.

3.1. Isolation of Placental Epap-1 Secreting cells.

Initial experiments were carried out to identify Epap-1 expressing cells in whole placental tissue. The cells in the placental tissue were disaggregated from placental tissue by treatment with trypsin and DNase-I. The cells were fractionated on 5-70% percoll gradient at 400 x g. There were three distinct cell populations separated at distinct densities as shown in **Figure 4.** The cells in the three bands were collected, washed and cultured in DMEM F12 complete medium (**Figure 5)**. After 48 hours, the growth of rapidly replicating cells was inhibited by addition of 5-fluoro deoxy Uridine (FDU) (**Figure 6**). The cells were treated with FDU for a period of 3 days. The cells that survived after FDU treatment were recultured in complete medium for 1 week. The non-adherent cells were separated and adherent cells were washed and continued to grow to form the confluent monolayer. Where as the non-adhering cells could survive for only 2 weeks. The supernatants of non adherent cells were negative for Epap-1. (**Figure 7)**

The adherent cells were continuously growing to form confluent monolayer. The culture supernatants (spent medium) were found to contain Epap-1 (**Figure 8).** Since these cells were isolated from first trimester tissue, we would like to examine the placental tissue region in which these cells are localized. Generally, the placental tissue collected during first trimester is composed of three morphologically distinct regions. (i) Amniotic sac, (ii) Hard tissue (decidualized endometrium) and (iii) Chorionic villous. These tissues were separated (**Figure 4)** and independently processed for the isolation of Epap-1 expressing cells (as described above). The morphology of isolated cells is shown in (**Figure 8 and 9).** Purification of Epap-1 from SNA affinity chromatography (**Figure 8**) show that Epap-1 is only present in culture supernatant of villous tissue. Further, the Eppa-1 was continuously expressed in these cells for more than 2 years **(Figure 10 Panel B).** The proteins were confirmed by western blot using Epap-1 specific polyclonal antibody Hence, Epap-1 expressing cells are present in villous tissue, which play a protective role in placenta (Kollmann *et al.,* 1996).

FIGURE 4

Isolation of Placental cells from MTP placental tissue.

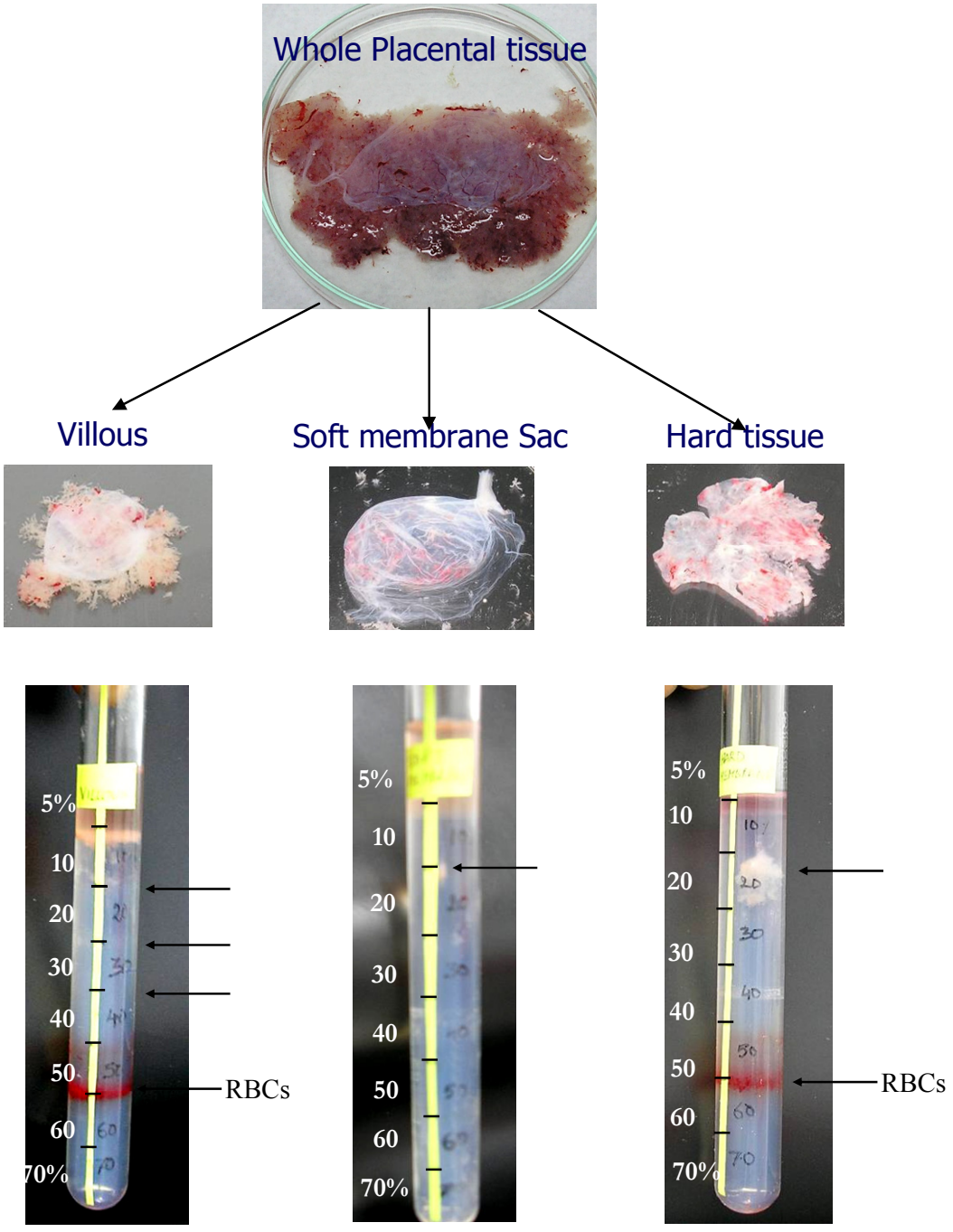
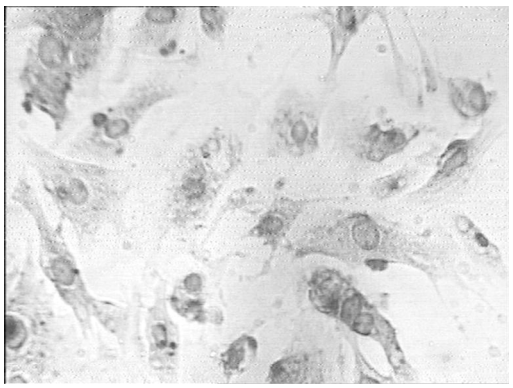


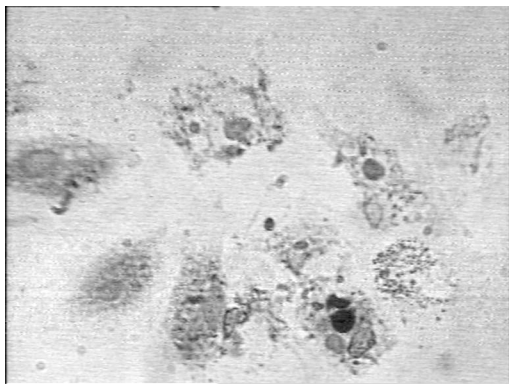
FIGURE 5

Morphology of placental cells isolated from different tissues

Villous tissue



Hard layer tissue



Soft membrane Sac

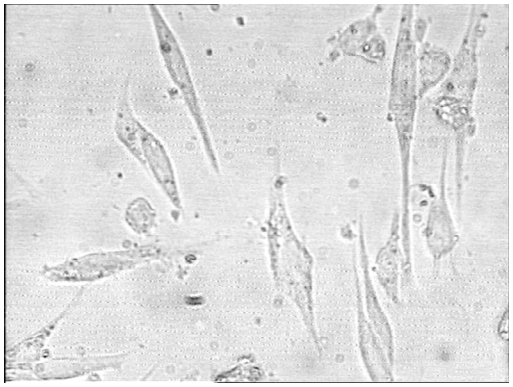
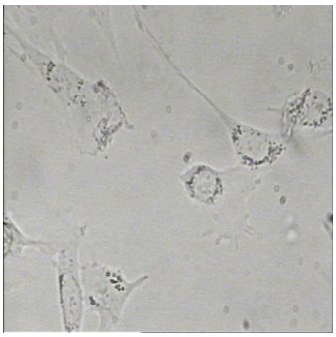


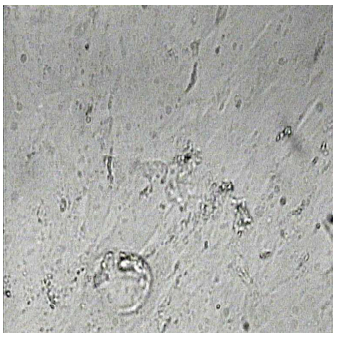
FIGURE 6

Placental cells treatment with 5-fluoro deoxy uridine (FDU).

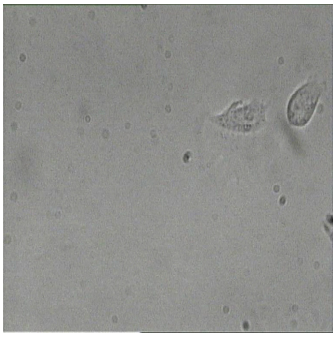
Adherent Placental Cells



Suspension Placental cells



Adherent Placental +FDU



Suspension Placental cells+ FDU

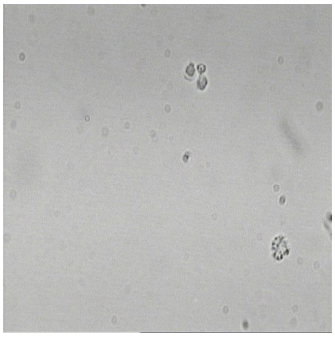


FIGURE 7

SNA affinity chromatography of culture supernatant from non-adherent placental cells

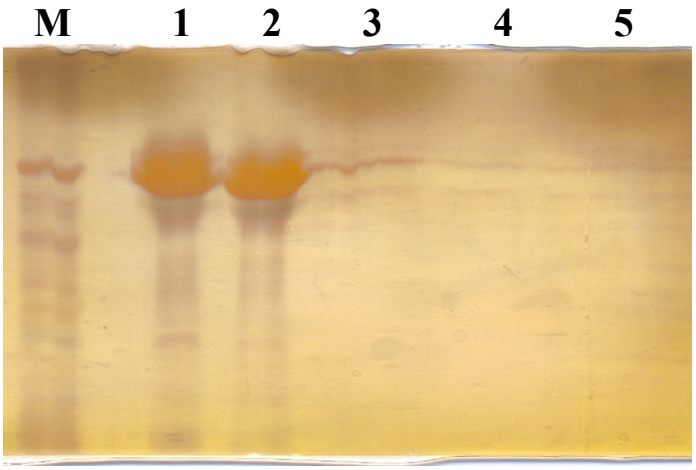
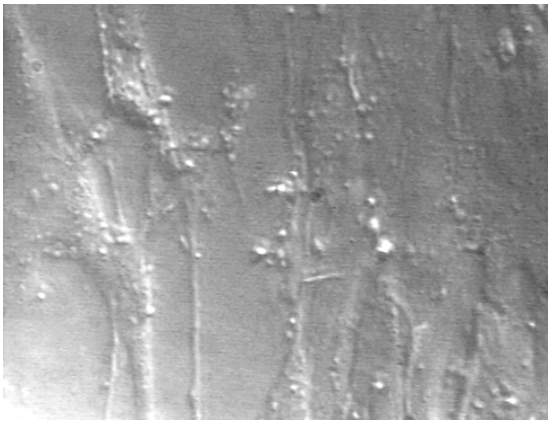


FIGURE 8

Pure cultures established

Differentiated Placental Cells + FDU (3 Months)

Monolayer



Multilayered

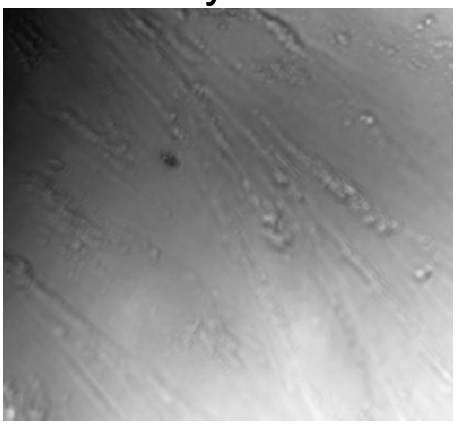
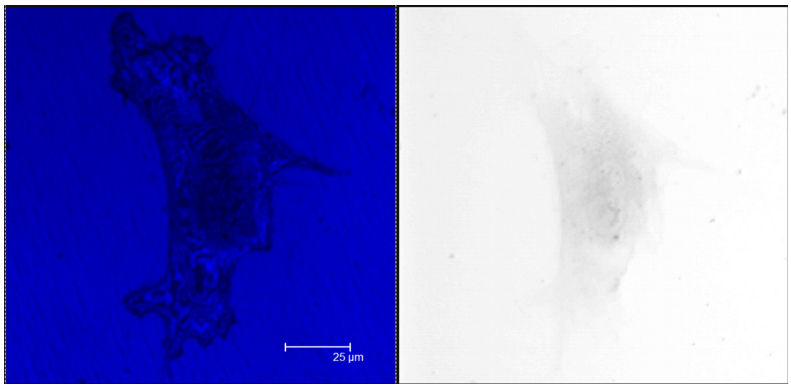


FIGURE 9

Adherent Placental Cells +FDU



Adherent pure culture of placental cells after 20 months

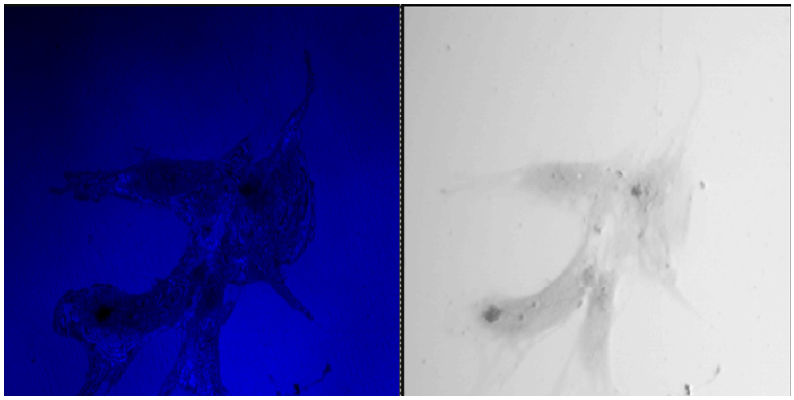
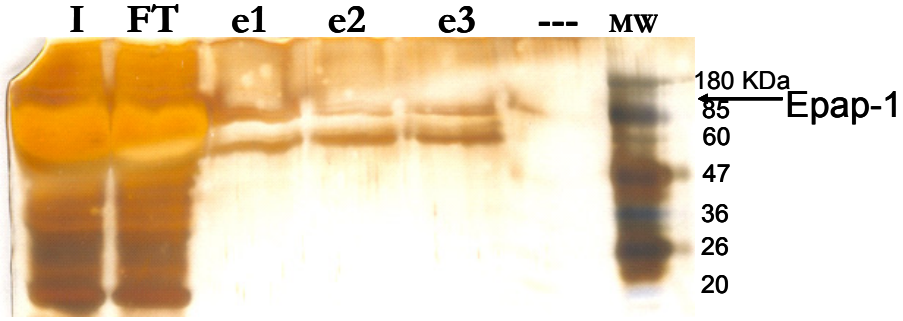


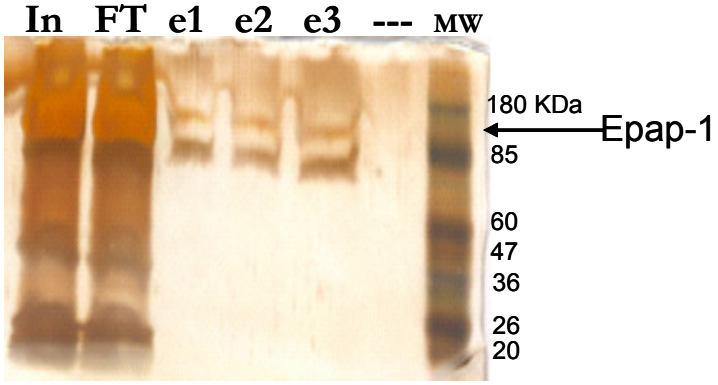
FIGURE 10

Purification of Epap-1 secreted by adherent placental cells using SNA affinity chromatography

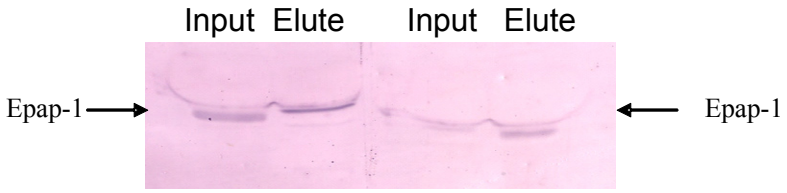
Panel A: Epap-1 from 3 month old placental cells



Panel B: Epap-1 from 16 month old placental cells



Panel C:Western Blot analysis with Rabbit anti-Human Epap-1 Ab



3.2. Characterization of isolated Placental cells:

To obtain pure cultures, these cells were serially diluted and assessed for expression of Epap-1. The least dilution at which the cells grow and produced Epap-1 was cultured to obtain uniform cell population.

These cells were characterized in terms of,

(i) Growth properties,

(ii) Morphology

(iii) Phenotype,

(iv) Efficiency of Epap-1 secretion and

(v) Anti-viral properties.

Growth properties: The cells in pure cultures isolated from villous tissue were growing continuously for the past two years. They show a doubling time of 5 days. These cells grow as a monolayer **(Figure 9**) with single nucleus. After reaching confluence, the additional cells were not disassociated; rather they grow in a multilayer morphology (**Figure 8**). The frozen cells in liquid Nitrogen in presence of DMSO could not be revived suggesting these cells are not immortalized or tumor origin cells. Further these cells don't need any extra additional nutrient supplements for adherence as well as continuous growth.

Morphology: The Epap-1 expressing cells were monitored by (giemsa stain) specific to cytoplasm. This stains cells cytoplasm with faint blue color. The results of these studies show that these cells have extended morphology with fiber like structures protruding on its surface. The cells contained single nucleus (**Figure 9**).

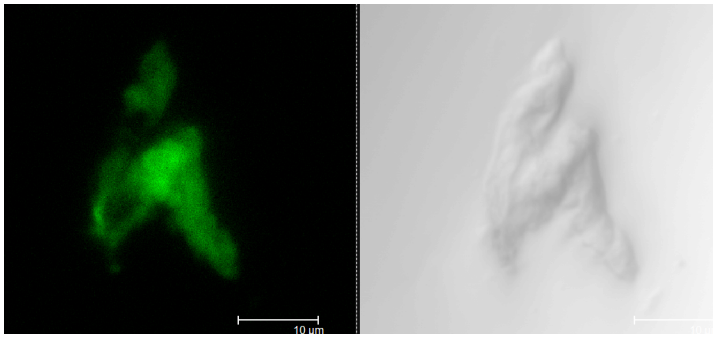
Phenotypic analysis: the phenotype of these cells was analyzed for expression of cell specific markers, Vimentin (specific to blood vessel endothelia), CD9 (specific to endothelia cells), Cytokeratin (specific for trophoblast cells), Epap-1 (specific Epap-1 protein) and human Chorionic Gonadotrophin (leutinizing hormone). The cells were grown on cover slips, fixed and treated with mouse anti-human IgG against vimentin, cytokeratin and CD9. The bound monoclonal antibody was detected with ant-mouse IgG conjugated to FITC. The stained cells were analyzed by confocal microscopy. The results (**Figure 11**) show that the cells were positive for CD9 and vimentin, and negative for cytokeratin suggesting that these cells are endothelial lineage. The cells were analyzed using polyclonal Epap-1 antibody and results (**Figure 12**) show that the cells are positive for Epap-1.

To confirm the above results the cells were also analyzed by cell specific markers using FACS analysis. The results in **Figure 13 Panel A and Panel B** show that the cells indeed are CD9 positive, vimentin positive, and cytokeratin negative. Further, the analysis of cells using polyclonal antibody against Epap-1 and monoclonal antibody against hCG showed that these cells expresses both the proteins. These results were consistant with the observations made in the confocal microscopy. Based on the characteristics these cells will be referred as Placental Epap-1 Secreting Endothelial cells (PESE cells).

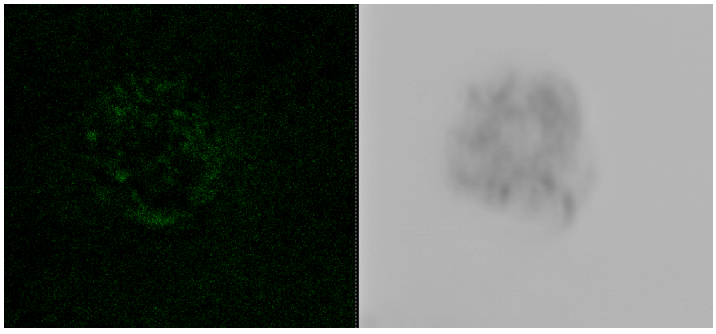
FIGURE 11

Characterization of Isolated Placental cells

Vimentin Positive



CD9 Positive



Cytokeratin Negative

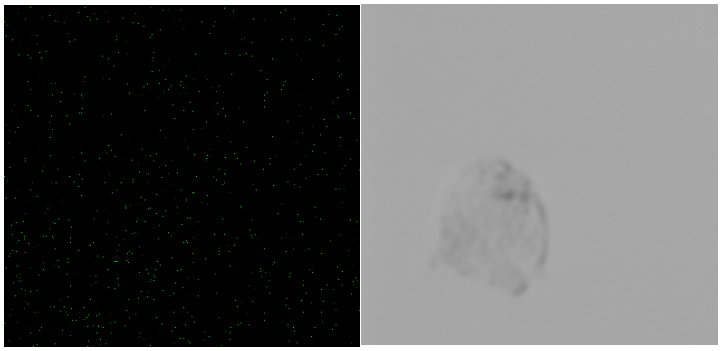


FIGURE 12

Epap-1 expression in cells is analyzed by RITC- Epap-1 antibody

Epap-1 POSITIVE

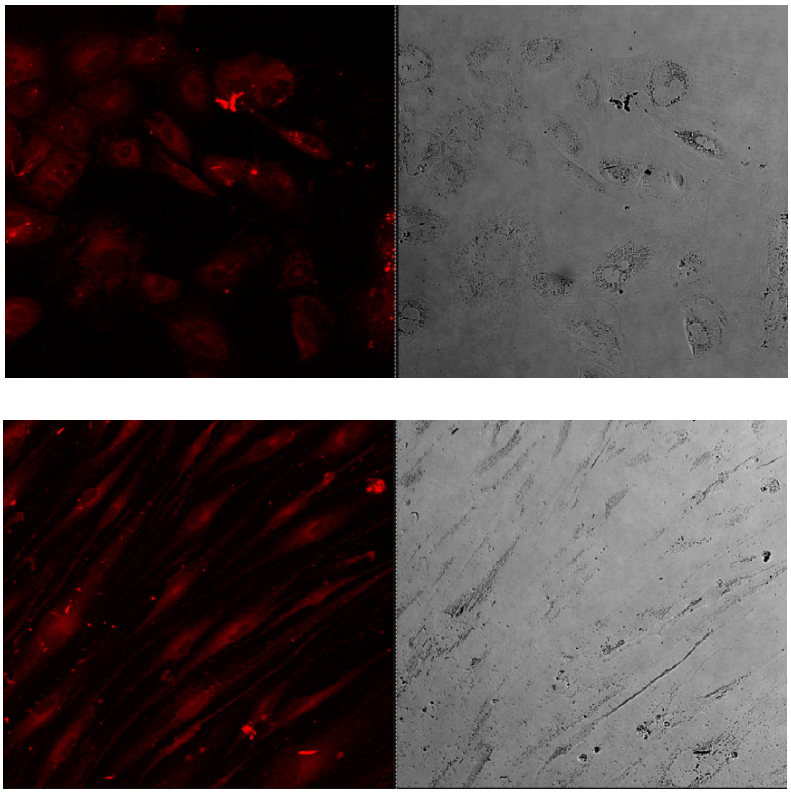


FIGURE 13

Panel A

FACS analysis of placental cells with cell type specific markers

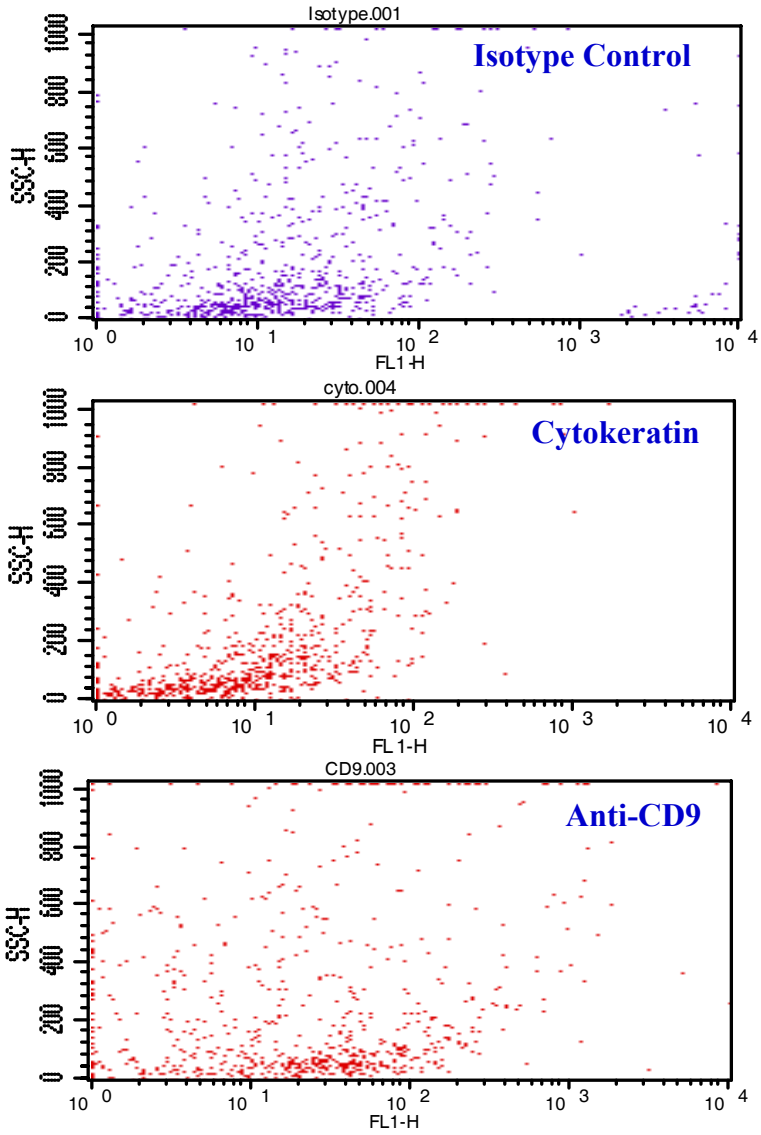
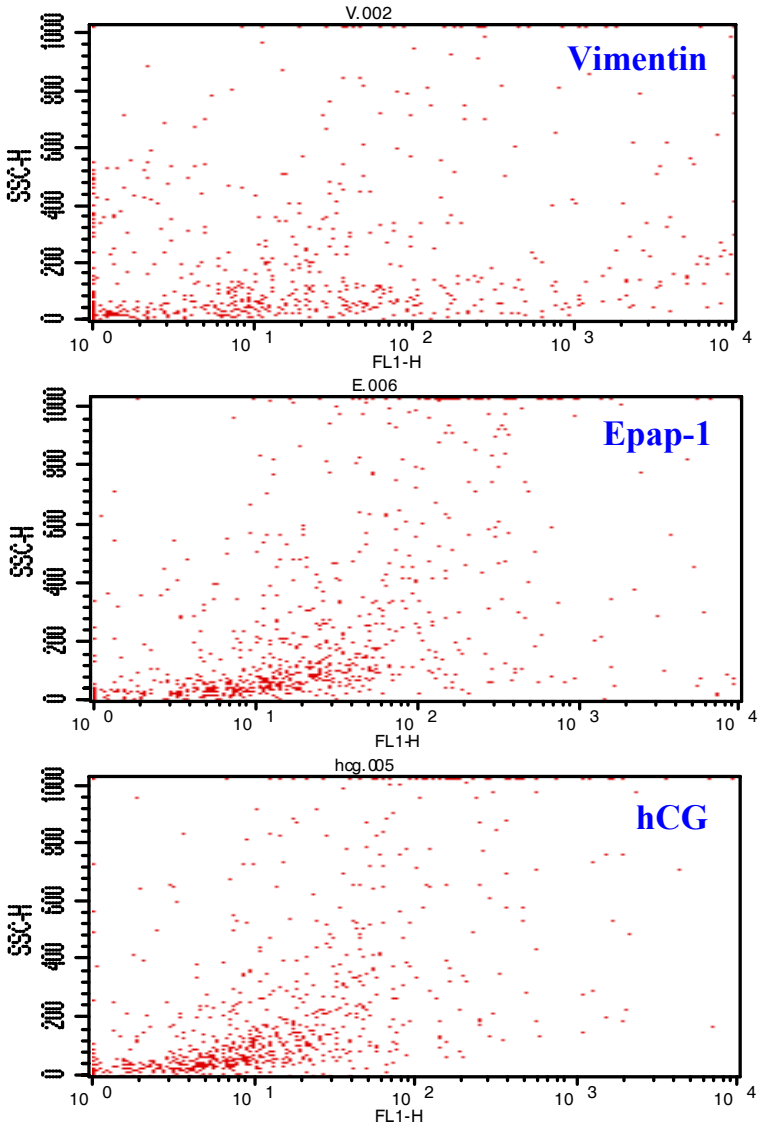


FIGURE 13

Panel B



3.3. Purification and characterization of Epap-1 from cell supernatant of Epap-1 secreting placental cells:

Epap-1 production by PESE cells: The biochemical characteristics of Epap-1 produced by PESE cells was assessed. The cells were grown for 4 days and the culture supernatant was filtered through 0.2μm filter. The proteins were ammonium sulphate fractionated at 0-60 and 60-80%. The 60-80% ammonium sulphate fraction was dialyzed and tested for its binding properties. The Epap-1 was purified by using *Sambucus nigra* affinity chromatography (as explained in methods). A 4 day culture supernatant from 5 million cells of PESE could produce 4μg of Epap-1**(Figure 8).** These results suggest that Epap-1 secreted from PESE cells retains SNA binding property. Further, we have examined the biochemical properties of purified Epap-1 from *Sambucus nigra,* in terms of binding to (i) Leupeptin Agarose matrix, (ii) Mono S ion exchange matrix, (iii) Phenyl Sepharose. The Results of these studies show that the purified Epap-1 from PESE cells possesses binding affinity to Leupeptin, Mono S and Phenyl-Sepharose **(Figure 14)** a property exhibited by native Epap-1 (purified from MTP tissue homogenate). But the protein eluted form Leupeptin, Mono S lost its anti-viral activity , where as the protein eluted form SNA and Phenyl Sepharose retained the anti-viral activity (**Figure 15**). These observations suggest the requirement of hydrophobicity for the activity of the protein. In conclusion the PESE secreted Epap-1 possesses similar biochemical properties as native Epap-1. **(Figure 16)**

FIGURE 14

Binding properties of Placental Epap-1

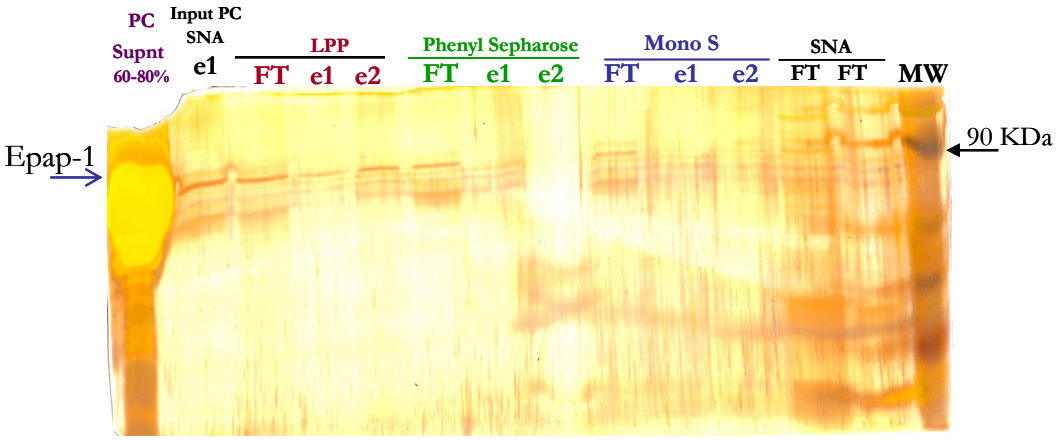


FIGURE 15

Antiviral activity of the PESE Epap-1 purified using SNA, Leupeptin, Mono S and phenyl Sepharose.

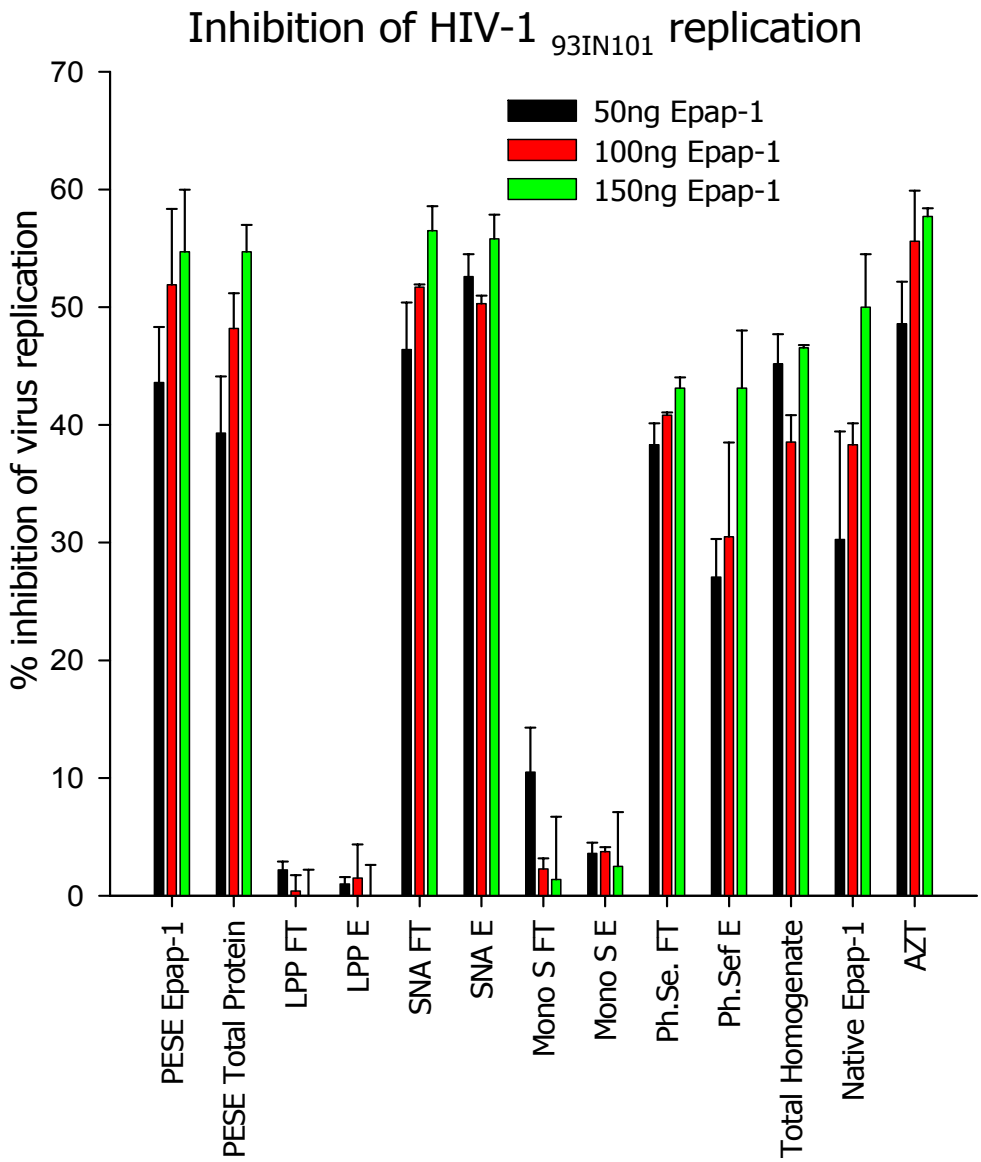
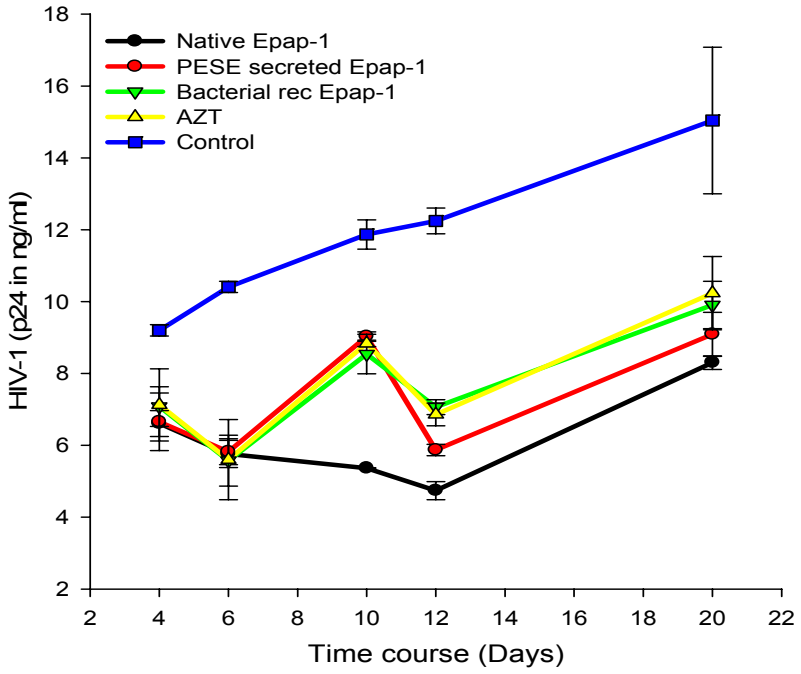


FIGURE 16

Protection of Placental cells from HIV-1 infection by PESE cells secreted Epap-1 and Bacterial recombinant Epap-1



3.4. Analysis of anti-viral properties of PESE cells

Antiviral property of PESE cells: Since these PESE cells are endothelial in origin and can produce significant levels of Epap-1, we investigated the potential role of PESE cells in regulating viral replication.

a). Endogenously produced Epap-1 can protect PESE cells from HIV infection: The dynamics of anti-viral potential of endogenously produced Epap-1 was monitored through challenging of PESE cells with virus at different time points in presence and absence of spent medium. The amount of virus replicated at day 4 was assessed.

To monitor the extent of protection provided by secreted Epap-1, we have challenged PESE cells with HIV-$1_{93IN101}$ in the presence and absence of spent medium. The infection was stopped at 1, 3, 6, and 12 hours. The cells were washed at indicated time points and recultured in fresh medium and harvested at day 4. The amount of virus replicated was quantified in terms of p24. The results in **Figure 16** show that infection is significantly inhibited in presence of spent medium that contained endogenously secreted Epap-1, suggests that endogenously produced Epap-1 can autoprotect PESE cells from viral infection.

To examine the dynamics of endogenous Epap-1 production, the PESE cells were washed and cultured in fresh medium and these cells were challenged with the virus. The infection was stopped at 1, 3, 6 and 12 hours and washed and replenished with fresh complete medium. The amount of virus replicated in terms of p24 was quantified at day 4. The results in **Figure 17 Panel C** show that in absence of spent medium containing secreted Epap-1, the PESE cells becomes sensitive to viral infection. Since the dynamics of secretion of Epap-1 *in vitro* from PESE cells were slower than the virus interaction, it took 12 hours to show 40%

protection of PESE cells form HIV-1 infection. We donot know if the dynamics of Epap-1 secretion in tissue and *in vivo* is different.

The above results suggest the auto-protective activity of secreted Epap-1 to the PESE cells from the HIV-1 infection.

b). Protective activity of endogenously Epap-1 secreting PESE cells to the coculture trophoblast cells:

Since secreted Epap-1 shown to protect PESE cells from HIV-1 infection, we would like to investigate whether the PESE cells with and without spent medium containing secreted Epap-1 can provide protection to the co-cultured trophoblastic cells form virus infection. Trophoblastic cells were isolated from placental tissue. PESE cells were incubated for a period of 4 days.

The PESE cells were co-cultured with trophoblastic cells in presence and absence of spent medium. These co-cultured cells were incubated with the virus. The amount of virus replicated at day 4, 6, 8 and 10 were analyzed. The results in **Figure 18** show that the virus replication in the co-cultured trophoblastic cells was inhibited by 40-50% when compared to that of the HIV-1 infected trophoblastic cells. Further the virus replication in the co-cultured cells is controlled over a period of 10 days. These results indicate the possible protective role of PESE cells against HIV-1 infection of trophoblastic cells

It has been shown that trophoblast cells *in vitro* are very sensitive to viral infection **(Tschering-Casper, 1999),** but the careful isolation of trophoblast cells from HIV-1 positive pregnant women showed negative for virus DNA **(Tschering-Casper, 1999)**, suggesting the existence of protective mechanism in the infected women during pregnancy that prevented the infection of trophoblasts and the fetus. Our results indicate reduced infectivity of

trophoblasts in presence of Epap-1 in coculture model. Since the present model involves cultured cells, the cells have a high virus encounter, in case of placental tissue; these cells may or may not be closely associated. If these cells are partitioned towards the maternal blood flow, the endogenously expressed Epap-1 may provide protection to various trophoblast lineage cells that invade the maternal decidua. This point out the possible contact of PESE cells to monocytes. To answer this, we have performed coculture experiments, where in PESE cells in presence and absence of endogenously secreted Epap-1 were cultured with PBMC and challenged with virus. The results (**Figure 19**) show that the endogenously secreted Epap-1 in spent medium of co-cultured PESE and PBMC can indeed inhibit viral replication in PBMCs suggesting a possible role of PESE cells in reducing the viral infectivity in PBMCs and such mechanisms may occur in placental tissue in down regulating viral load. Such reduced viral load is frequently shown to be associated with the seronegativity of the offspring. (Coll et al., 1997)

PESE cells in presence of secreted Epap-1 can reduce viral replication in chronically infected SupT1 cells:

HIV-$1_{93IN101}$ chronically infected SupT1 cells were co-cultured with PESE cells, in presence and absence of spent medium containing secreted Epap-1. The amount of virus replicated at day 4 was quantified. The results in **Figure 20** shows that co-culturing of chronically infected SupT1 cells with PESE in presence of secreted Epap-1 shows significant reduction of virus turnover suggesting that the PESE cells can reduce the virus turnover in chronically infected T-cells in the vicinity. Thus may contribute in reduction of circulating viral load in placenta.

In summary, the endogenously secreted Epap-1 can reduce HIV-1 replication in PESE cells itself, as well as other cells in the vicinity namely trophoblast and PBMCs. Hence, PESE cells could be recruits of innate immunity that express Epap-1 as one of the candidate molecule for decreasing the viral load to protect the fetus from virus infection.

The hypothetical model that we propose on the Epap-1 mediated protection of viral infection is that PESE cells are of endothelial cell lineage that may be present at the inner lining of blood vessel that supply nutrients from the mother to the fetus. We propose the following model (**Figure 21**).

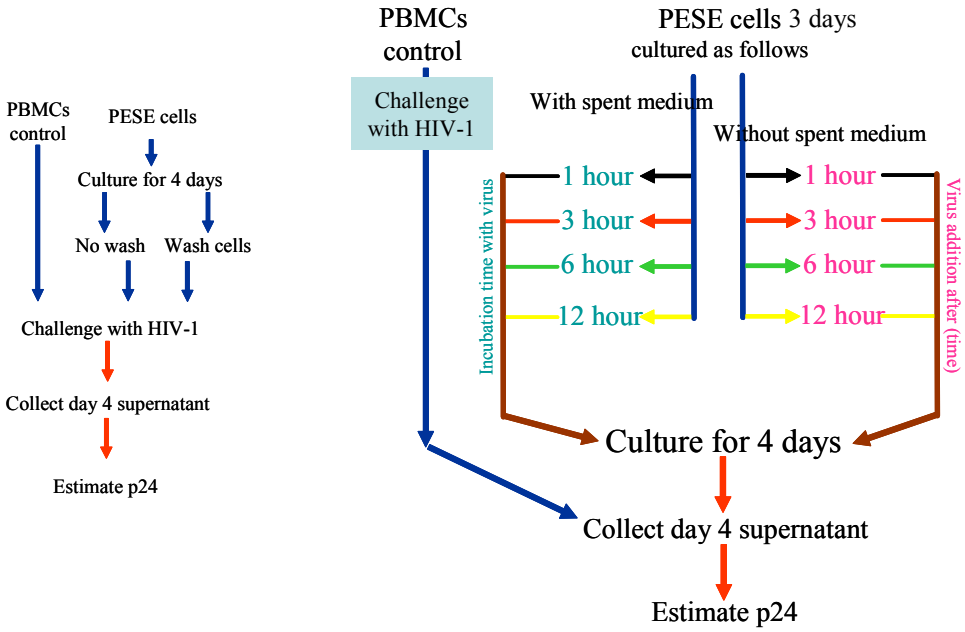
The placenta is a unique semi-autocrine organ, protecting the semi-allogenic fetus by evading the immune recognition from the maternal immune system with HLA-G expression and maintaining predominantly innate immunity. The cell mediated immunity is suppressed with recursion of suppressor T and NK cells. Thus, preventing immune recognition of fetus by the mother and at the same time protecting it form pathogenic infections. The decidualized placenta is known to produce various innate immune factors (Table 1). Though trophoblast are susceptible to HIV-1 infection *in vitro*, based on the reports (Tschering-Casper, 1999), that careful isolation of trophoblast from HIV-1 seropositive individual were found to be negative for HIV-1 *in vivo*, suggests the presence of certain innate immune factors protecting the fetus from viral infection *in vivo*. Our investigation (Kondapi *et al*., 2002) show that Epap-1 (Early pregnancy associated protein-1) is produced during first trimester of pregnancy is anti-HIV-1 active against various HIV-1 strains tested. As the Epap-1 secreting placental cells are of endothelial origin and in the decidualized placenta the endothelial cells are found in the blood vessel lining. We propose that the isolated placental endothelial cell secreted Epap-1 could be protecting the cells in the placental milieu (trophoblast, endothelial cells etc.) by being located as a cell lining in various regions of placenta especially as inner layer

of maternal blood vessels that are involved in transport of blood and nutrients to the fetus and certain critical levels of Epap-1 might be required to neutralize the viral load encountered. Hence, Epap-1 secreting placental cells can be a new cell based therapeutic agent against HIV-1 infections. Further investigations are required to confirm this model.

FIGURE 17

The dynamics of virus neutralization by endogenously produced Epap-1 of PESE cells

Panel A



Panel B

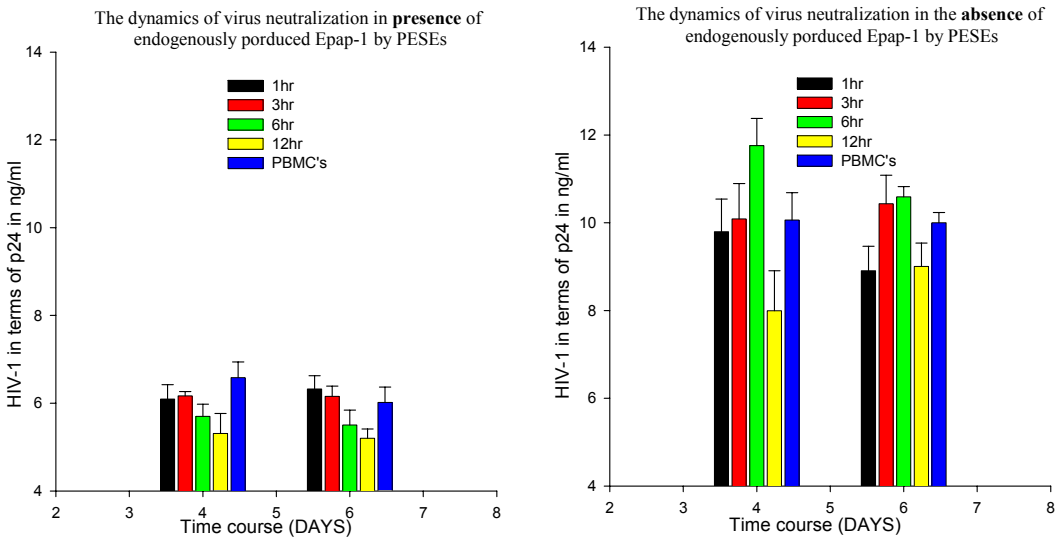


FIGURE 18

PESE cells can protect Trophoblast cells from HIV-1 infection

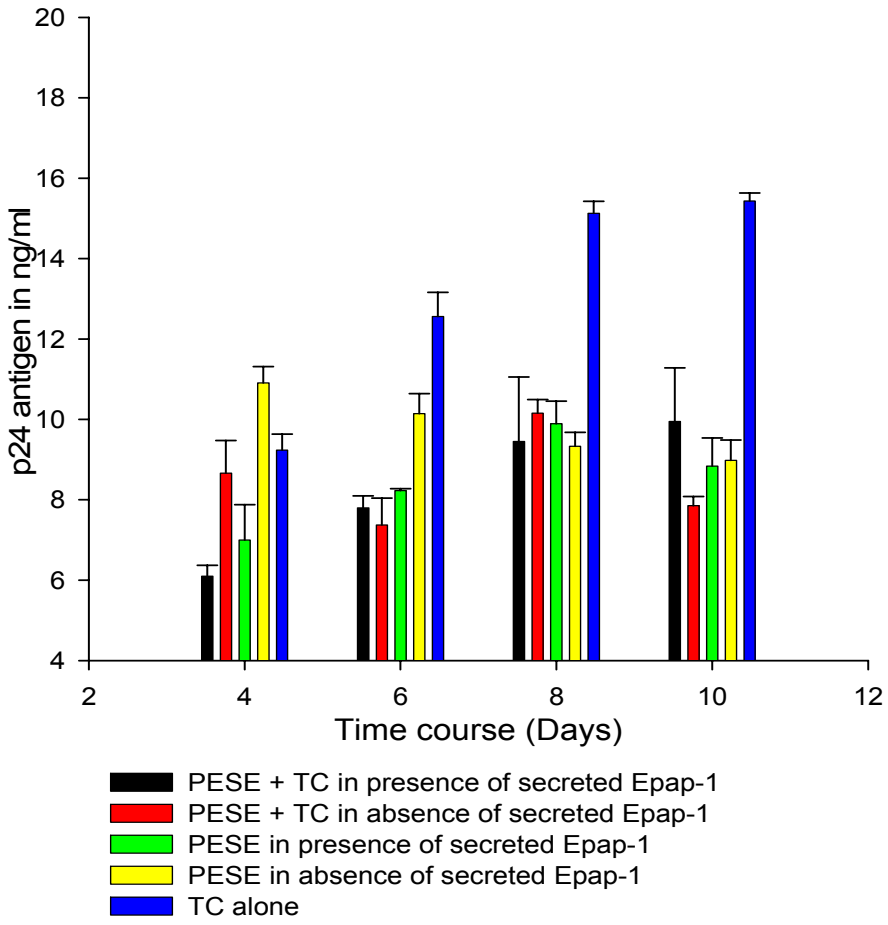


FIGURE 19

PESE cells can protect PBMCs from HIV-1 infection

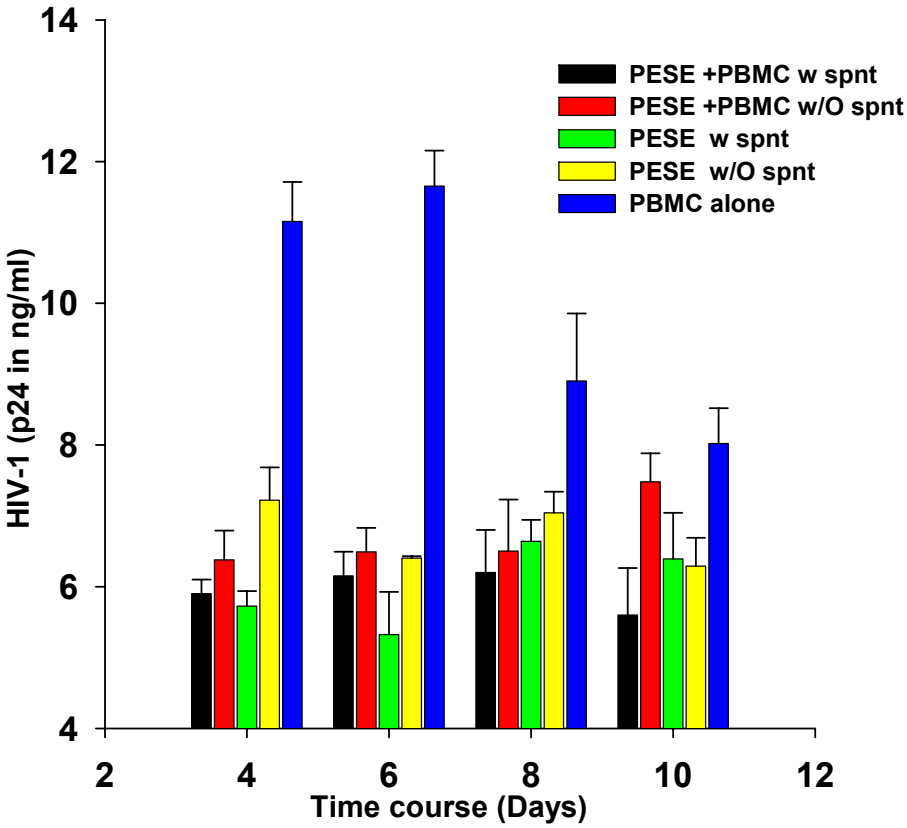


FIGURE 20

PESE cells can protect chronically HIV-1_{93IN10}1 infected SupT1 cells

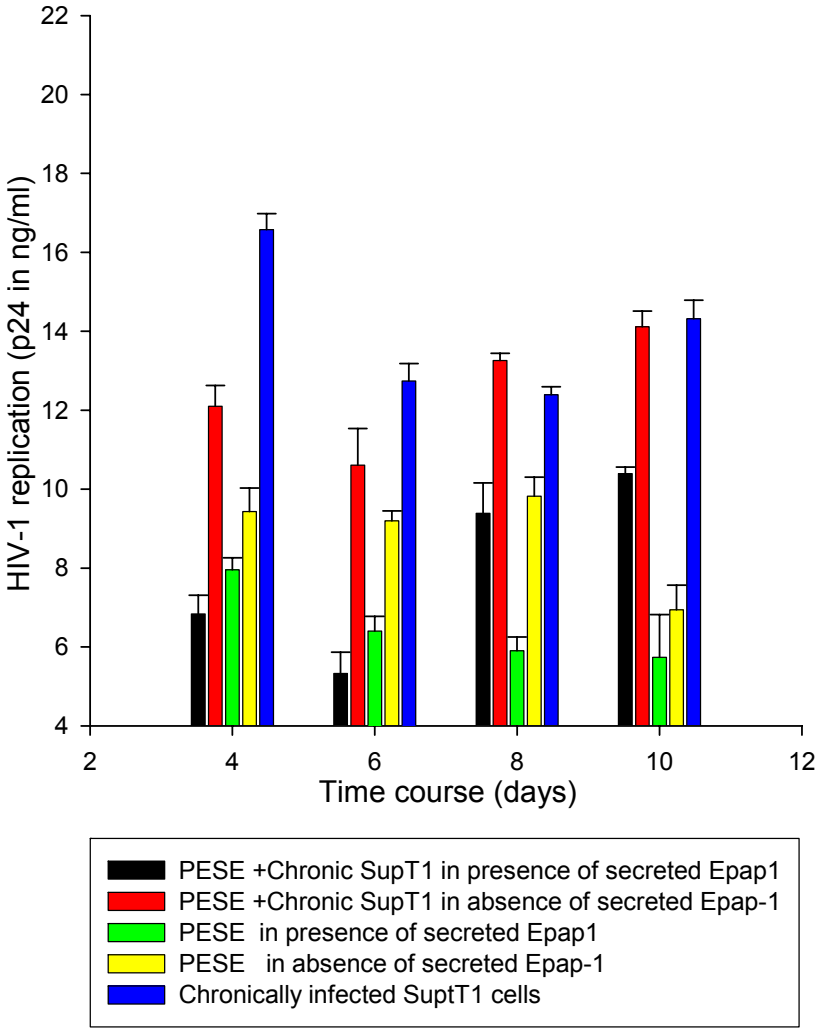
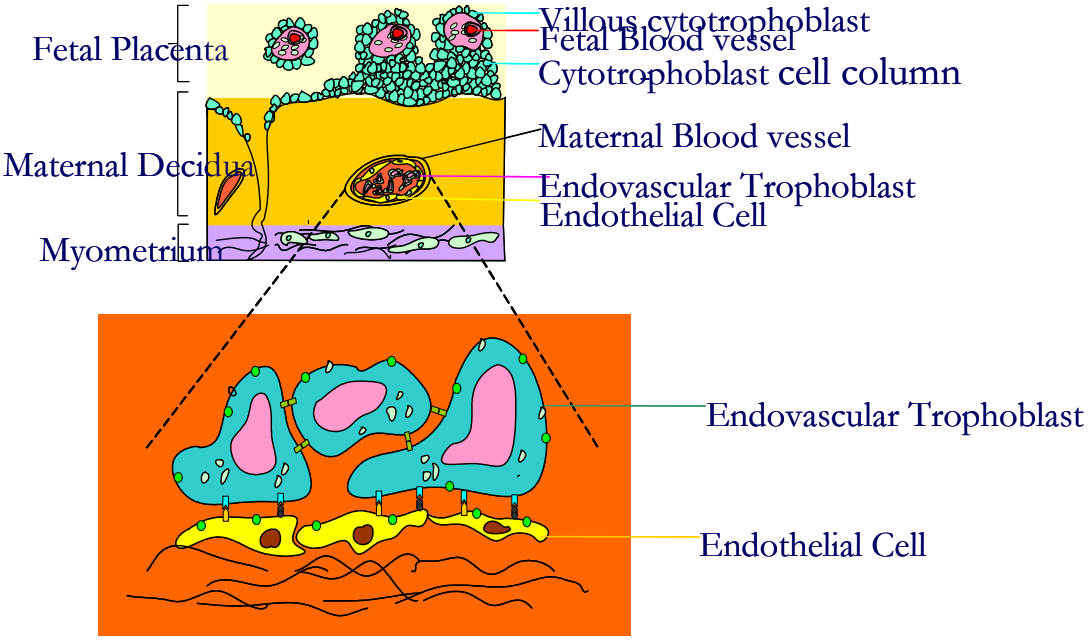


FIGURE 21

A Hypothetical model on localization of PESE cells



Part II

Cloning and characterization of the anti-viral activities of Epap-1 expressed in bacteria.

4. Introduction

The retrovirus, HIV-1 consists of an outer envelope derived from host cell lipid bilayer and virus encoded glycoproteins (Kwong *et al*., 1998) encasing the viral RNA genome. HIV-1 infects cells expressing the primary receptor CD4+ and co-receptor (CXCR4 /CCR5)in the T cells, macrophages etc (Gallo *et al*., 2003). The critical HIV-1 viral entry process is primarily mediated by the viral trimeric envelope glycoprotein gp120-gp41 interaction with the host cell CD4 receptor followed by interaction with coreceptor (Berger *et al.,* 1999; Doms, 2000). This interaction triggers conformational changes in surface protein (Kwong *et al*., 1998) causing disassociation of gp120 from gp41. The gp41 then forms fusion peptide with pre-hairpin configuration of the ectodomain (Isabel Muñoz-Barroso *et al.,* 1998; Weissenhorn *et al*.,1999 and Zwick *et al*., 2005) to be inserted into the host cell membrane for the virus –cell membrane fusion and release of viral genome into host cell cytoplasm.

HIV enters macrophages and T cells by recognition and binding of envelope to CD4 receptors following by fusion with the cell membrane mediated co-receptor and the release of the HIV capsid into the cell. The HIV RNA and various enzymes, including but not limited to reverse transcriptase, integrase and protease, are injected into the cell. The RNA is transcribed into DNA by reverse transcriptase leading to synthesis of viral cDNA which integrates into host chromosome (Zheng *et al.*, 2005).

HIV-1 infection is associated with a progressive CD4 down regulation and loss of CD4+ T cells (lymphocytes). This rate of loss can be measured and is used to determine the stage of infection. The loss of CD4+ T cells is linked with an increase in viral load. HIV plasma levels during all stages of infection range from just 50 virions to 11 million virions per ml (Piatak *et al.,* 1993). There are four stages of HIV infection: primary infection (or viremia or acute infection) which progresses over time to clinical latency (where the virus is a provirus inside

monocytes) and then to symptomatic HIV infection, and finally, AIDS which is identified on the basis of certain co-infections. The decreased immune competence leads to cytomegalovirus infection of the retina, Pneumocystis carinii infection of the lungs, or tumours like Karposi's sarcoma or non-Hodgkin's lymphoma. HIV viral load is an excellent predictor of progression to AIDS. Viral load and CD4 count can predict the success and failure of antiretroviral therapy (Hughes and O'Brien *et al.*, 1997). Different classes of antiretroviral drugs act at different stages of the HIV life cycle. Combination of several (typically three or four) antiretroviral drugs is known as Highly Active Anti-Retroviral Therapy (HAART).

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The rationale of present study is to examine whether glycosylation is essential for the anti-viral activity of unglycosylated Epap-1. The best method for preparation of unglycosylated form of Epap-1 is through expression of Epap-1 cDNA in bacterial expression system. Since bacterial expression system is devoid of post translational modification through glycosylation, the protein expressed in this system is truly unglycosylated form. Further such study also can indicate if peptide portion alone is sufficient for its anti-viral activity.

5. Methodology

MTP placental tissue cDNA library was constructed,Epap-1 cDNA was isolated and cloned into TA vector by Roda rani et al, (2006). We have taken the TA vector and the insert was removed from the TA clone by restriction digestion and cloned into bacterial expression vector pET32 HTa.

5.1. Isolation plasmid DNA by alkaline – SDS lysis method:

The positive transformed DH5α cells with insert DNA was cultured (500 ml) and harvested at 4000 rpm for 10 min. The cell pellet was suspended in 20 ml of chilled PI buffer (lysis buffer: 50 mM glucose, 25 mM Tris-HCl P^H8, 10 mM EDTA) and incubated on ice for 10 min. To the above solution, 40 ml of PII (0.2N NaOH, 1% SDS) was added, mixed and incubated at 37^0C for 15 min. To the above solution, 25 ml of 3M NaOAc pH5.2 was added, gently mixed and incubated on ice for 10 min.Spinned at 12000 rpm in rotor for 20 min at 4^0C and supernatant was collected through glass wool. To the supernatant, 40 ml of solution containing phenol, chloroform, isoamylalcohol at a rotio of 25:24:1 was added and vortexed for 30-60 sec. Spinned at 12000 rpm in rotor for 10 min at 4^0C and aqueous phase was collected. To the supernatant 0.8 volume of isopropanol was added and incubated at 37^oC for 5 min. Spinned at 12000 rpm in rotor for 10 min at 4^0C and supernatant was discarded. The pellet was washed with 70% ethanol and repelleted at 12000 rpm/ 10 min/37^oC. The pellet was air dried and dissolved in 20 µl of water.

5.2 Cloning of insert DNA into pET 32 HTa vector:

The His-Tag vector (HTa) was chosen based on the cDNA sequence analysis, ORF and orientation. 1µg of pET32 HTa vector and 3 µg of TA vector with insert DNA isolated (as above) was restriction digested with Kpn I (10 U) and Sac I (10 U) restriction enzyme in buffer (containing1 mM Tris,1 mM $MgCl_2$,100 µM DTT pH 7 and BSA at 100 µg/ml) and

the reaction mixture was incubated at 37^0C for 1 hr. The digested DNA was analyzed on 1% agarose gel electrophoresis and ethidium bromide stained, visualized under UV light. The linearized HTa vector and insert DNA was purified by gel extraction using qiagen gel extraction kit .The linearized HTa vector was ligated with insert DNA at 1: 3 ratio by T_4 DNA ligase (3U) and 10x ligase buffer. (20 mM Tris – HCl pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP). It was incubated at 16^0C for 16 hrs. This ligation mixture was used to transform E.coli, DH-5 α cells. The selected transformed colonies were analyzed for the insert DNA by colony cracking and PCR using M13 primers. The confirmed colonies were subcultured and the recombinant HTa plasmid with insert DNA was isolated and was used to transform BL 21plyse E.coli cells. The transformed cells were used to express protein by IPTG induction.

5.3. Expression of His –tagged protein in *E.coli* Bl-21 and purification of expressed protein using Ni column chromatography:

The recombinant pET32 HTa vector transformed *E.coli* BL21 cells were incoculated into 10 ml of LB (ampicillin, 100 µg/ml) and incubated at 37°C/ 150 rpm overnight. This was used as seed culture to inoculate 100 ml of prewarmed media (with ampicilin, 100 µg/ml) and grown at 37°C with vigorous shaking until an OD_{600} of 0.6 is reached (60 min). Collected 1 ml sample. The IPTG to a final concentration of 1 mM was added for induction of pET32 HTa Vector for expression of protein. Incubation of the cultures was continued for an additional 4–5 h. During this time the IPTG induced expression of insert DNA. 1 ml of aliquote was collected. The cells were harvested by centrifugation at 4000 x *g* for 20 min. Freeze the cells in dry ice–ethanol or liquid nitrogen, or store cell pellet overnight at –20°C. Thaw the cell pellet for 15 min on ice and resuspended the cells in lysis buffer at 2–5 ml per gram wet weight. Add lysozyme to 1 mg/ml and incubate on ice for 30 min. Sonicate on ice

using a sonicator equipped with a microtip. Centrifuge the lysate at 10,000 x *g* for 20–30 min at 4°C to pellet the cellular debris. To 4 ml of supernatant, a 1 ml of the 50% Ni-NTA slurry was added and mixed gently by shaking at 4°C for 60 min. Load the lysate–Ni-NTA mixture into a column with the bottom outlet capped. Remove bottom cap and collect the column flow-through. Wash twice with 4 ml wash buffer; collect wash fractions for SDS-PAGE analysis. Step elute with 0.5 ml elution buffer. Collected elutes were analyzed by SDS-PAGE.

5.4. Infection Assay:

One million/ml of SUP T1 or 2 day PHA-stimulated peripheral blood mononuclear cells with 100% viability in RPMI 1640, 0.1% FBS were seeded in four 12 well plates. Increasing concentration of Epap-1 (250 ng, 500 ng, 1 μg) were added to the cells and were infected with HIV-1 $_{93IN101}$, HIV-1 $_{CEM\ -50}$, HIV-1 $_{UH1}$ and HIV-1 $_{MN}$ each at a final concentration of virus equivalent to 2 ng of p24 per ml. The infected cells were incubated at 37 °C and 5% CO_2 incubator (Forma Scientific, USA) for 2 h. After 2 h, the cells were pelleted at 350 x g for 10 min, supernatant was discarded and cells were washed with RPMI 1640 containing 10% FBS and were resuspended in the same medium with increasing concentrations of native and recombinant Epap-1 and were incubated for 96h. The supernatants were collected after 96h and analyzed using p24 antigen capture assay kit (SAIC Frederic). The infection in the absence of Epap-1 was considered to be 0% inhibition. AZT was taken as positive control. In the infection conducted with PBMCs 10 U /ml of IL2 is added. The data given is an average of three independent experiments in the triplicates.

5.5. Analysis of action of recombinant Epap-1 on CD4-gp120 interaction:

Mouse monoclonal anti-human gp160 antibodies spanning different regions of HIV-1 envelope were added into 96 well RIA plate wells at 10 ng per well in PBS, the plates were incubated overnight. Following day the wells were blocked with 3% BSA for 2 hours at 37^{o}C, binary complexes containing gp160-Epap-1 were formed by incubation of gp160 in PBS with increasing concentrations of Epap-1 at 37^{o}C for 1 hour. Binary complexes were captured with gp160 monoclonal antibody pre-coated wells (as above) and incubated for 1 hour at 37^{o}C. The unbound complexes were removed by washing thrice with wash buffer. Captured binary complexes were probed for the Epap-1 using 10 ng of affinity purified Rabbit polyclonal anti-human Epap-1 antibody by incubating for 1 hour at 37^{o}C and wells were washed thrice with wash buffer. Bound rabbit polyclonal was probed with 1:2000 dilution of Goat anti-rabbit IgG-peroxidase antibody by incubating at 37^{o}C for 30 minutes, the wells were washed thrice with wash buffer and developed with TMB substrate system. The reaction was stopped after 30 min with 1N HCl and plates were read at 450 nm. Each experiment was done in triplicates and average and standard deviations were calculated.

Assay for ternary complexes of gp160-CD4-Epap-1: 10 ng of gp160 and 10 ng of CD4 was incubated with increasing concentration of Epap-1 in PBS at 37^{0}C for 1 hr. The ternary complexes were captured using mouse anti-human gp160 antibodies and Epap-1 in the captured ternary complexes was detected using affinity purified rabbit anti-human Epap-1 polyclonal antibody and analyzed as described Epap-1-gp160 binding assay.

5.6. Cell Fusion Assay

The cell fusion assay brings the actual membrane fusion process that is taking place when the HIV envelope membrane fuses to CD4 bearing T cell membrane for insertion of viral genome into cell. In the assay, the HIV-1 $_{IIIB}$ Env-expressing HL 2/3 cells previously stained with the cytosolic dyes Calcein AM (Molecular Probes, Inc., Eugene, OR) and SupT1 cells with calcein blue AM (Molecular Probes, Inc., Eugene, OR) are co cultured for the cell-cell fusion reaction. The dyes are cleaved inside the cell by nonspecific esterase's, which convert the colorless calcein AM into membrane impermeable fluorescent calcein green. Cell fusion is monitored using dye redistribution under fluorescent microscope as described previously (Yang et al, 2003).

Calcein AM labeling (excitation/emission 496/517): HL2/3 cells expressing HIV-1 $_{IIIB}$ gp120 on surface was incubated with 0.5 μM of calcein AM in DMEM +10 % FCS for 1 hour at 37^0C and 5 % CO_2 incubator (Forma Scientific, USA) for dye uptake. The cells were washed and incubated in fresh medium for 30 minutes at 37^0C, again washed and resuspended to 1 million cells/ml.

Calcein Blue loading (excitation/emission 354/469): Sup T1 cells were incubated with 20 μM of Calcein blue RPMI +10% FCS for 1 hour at 37^0C/ and 5 % CO_2 incubator for dye uptake. The cells were washed and incubated in fresh medium for 30 minutes at 37^0C, again washed and resuspended to 1 million cells/ml.

Fusion assay: The envelope (gp120-41) expressing cells HL2/3 and $CD4^+$, CXCR4 expressing SupT1cells were loaded with cytosolic dyes as described above. Both cells were co-cultured at 1:1 ratio in complete medium for 2 hours at 37^0C and 5% CO_2 incubator. The fusion inhibition was checked in presence of Epap-1 at 250 ng/ml. The cell fusion in the absence of Epap-1 was considered to be control cell fusion and in presence of CD4 reactive

(SIM 4) Mab was considered as positive inhibition control. Fluorescent images were acquired Leica Fluorescence microscope and Leica confocal Microscope.

5.7. Localization of Epap-1 and mapping the epitopes of HIV-1 gp120 involved in interaction with Epap-1

5.7.1. RITC labeling and binding analysis of Epap-1: The recombinant Epap-1 was labeled with rhodamine isothiocyanate (RITC) following Aaron Kantor method, by incubating with carbonate buffer pH 9.2 at 4^{o}C overnight on a rotator-coupler. The labeled proteins were immediately dialysed against PBS pH 7.3. The molecular anti-viral activity of the RITC Epap-1 during the course of cell fusion reaction as described above in methods was monitored for different time points. The cell fusion assay was performed as described in presence of RITC native and recombinant Epap-1.

Further the binding and affinity of RITC Epap-1 to envelope expressing HL 2/3 cells were studied in presence of (1) soluble CD4 (2) soluble gp120 (3) whole gp120 antibody ID6 .

5.8. Assays for Epitope exposure pattern of HIV-1 gp120 during cell fusion reaction (Antony *et al.,* 2005)

The epitope/s of gp120 being exposed during the envelope mediated fusion reaction in the presence of Epap-1 was monitored through the binding affinity of anti-HIV-1 gp120 specific monoclonal antibodies. The HIV-1 IIIB Env-expressing HL 2/3 cells (0.5 x 10^6) were plated at the bottom of 35 mm wells and incubated with SupT1 (0.5 x 10^6) cells in presence of 250 ng/ml native and recombinant Epap-1 at 37 °C and 5 % CO_2 incubator (Forma Scientific, USA) for a desired time in the range of 0-45 min. After timed incubation, the cells were immediately placed on ice to stop the fusion processes. Cells were washed

twice with ice-cold washing buffer (D-PBS with calcium and magnesium, containing 0.1% bovine serum albumin and 0.02% sodium azide). Nonspecific Fab binding was blocked with blocking solution (2% normal goat serum in D-PBS) containing 1 μg/mL unconjugated goat anti-mouse Fab for 30 min on ice. Then, the cells were washed twice with the washing buffer, once with 2 % goat serum, and blocking solution supplemented with various epitope specific mouse anti-HIV-1 gp120 Monoclonal antibodies (1/1000, 0.75 μg/ml) of the {C2 reactive (B32), C5 reactive 670-30D, V3 Loop reactive (III-V3-21), V3 domain reactive (257-DIV), CD4 reactive (SIM4)}. Incubation continued for 1 h on ice. The cells were washed twice with washing buffer and once with blocking solution. Incubated with the secondary antibody, 1 μg/ml FITC conjugated goat anti-mouse Fab, (BD Bioscience) in blocking solution. Incubation continued for 1 h on ice. The cells were washed thrice and covered with 1 ml/well washing buffer prior to microscopic observation.

5.9. Cell based ELISA Assay method (Zhao et al, 2003)

NIH 3T3 CD4 cells or NIH 3T3 CD4, CXCR4 cells were grown in a 75 cm^2 flask. The cells were grown in DMEM medium, supplemented with 10 % fetal calf serum, L-glutamine, sodium pyruvate and penicillin/streptomycin at 37 °C in 5 % CO_2 in air atmospheres. Cells at the log growth phase were harvested from cultures, washed twice in phosphate-buffered saline (PBS) and suspended to the indicated cell density. 200 μl of cell suspension was added to each well of a 96-well ELISA plates (poly-D-lysine coated). The plate was centrifuged at 1000 rpm for 10 min using a rotor specifically designed to carry micro-titer plates (Heraeus). The supernatants from the wells were carefully removed. The plate was fixed with 4% formaldehyde. After washing with PBS containing 0.05 % Tween-20 (PBST), non-specific binding sites were blocked by incubation with 200 μl of blocking buffer (PBS containing 3 % BSA and 2 % NGS) for 30 min at 37 °C. After washing, the cells were

incubated with 50 µl of HIV-1/gp120 complexed with Epap-1(0, 50,100,150ng) for 1 hr at 37 °C. After washing, 50 µl of various epitope specific gp120 Monoclonal antibodies (1/1000, 0.75 µg/ml) {(C1 reactive (B2), C2 reactive (B13), C5 reactive 670-30D, V2 domain reactive 697-30D,V3 Loop reactive (III-V3-21), V3 domain reactive (257-DIV), (III-V3-13), V4 domain reactive (B15), CD4 reactive (SIM4)}were incubated for 1 hr . After washing, 50 µl of the goat-anti-mouse IgG conjugated to peroxidase (1/1000, 0.75 µg/ml) was added and incubated for 1hr min at 37 °C. The plate was washed and TMB substrate was added (50 µl) and incubated at room temperature. The reaction was stopped by adding 50 µl of 2N H_2SO_4. The absorbance was read using an ELISA plate reader (Softmax 190, Molecular Devices) at 450 nm.

6. Results and Discussion

The objective of present study is to express Epap-1 cDNA in bacterial system and characterize its anti-viral activity. This would examine if the glycosylation of Epap-1 is required for its antiviral activity and as well as if peptide portion of Epap-1 alone can exhibit the anti-viral activity.

6.1. Cloning and expression of Epap-1 in bacterial system:

Roda Rani *et al.*, prepared the cDNA of Epap-1 from placental MTP cDNA library. The pET 32 HTa bacterial expression vector was chosen based on the DNA sequence analysis, ORF and orientation of Epap-1 cDNA. The pET 32 HTa vector and the insert DNA in recombinant TA vector was digested with Kpn I and Sac I restriction enzymes. The digestion mixture was separated on 1% agarose gel (**Figure 22**). The linearized pET 32 HTa vector and insert DNA with Kpn I and Sac I cohesive ends was purified by gel extraction using quiagen gel extraction kit. The linear pET 32 HTa vector was ligated with insert DNA at a ratio of 1:3 using the T4 DNA ligase enzyme at 16^0C.

The DH5α cells were transformed with ligation mixture using $CaCl_2$-heat shock method and plated on LB agar plates (with ampicillin).The transformed colonies were analyzed for the presence of insert by colony cracking followed by PCR analysis with M13 primers **(Figure 23)** . The positive colonies were cultured and the recombinant HTa plasmid with insert DNA was isolated (**Figure 24**). *E.coli* BL21 p-lyse cells were transformed with the isolated recombinant DNA. The transformed BL 21 p-lyse cells log phase culture was induced with 1mM IPTG for the expression of recombinant Epap-1 protein with His-Tag. The His-Tag recombinant protein was purified using Nickel affinity chromatography. The results in **Figure 25** show that there is significant expression of recombinant protein**,** which was confirmed by western blotting anti-HisTag monoclonal antibody and Epap-1 polyclonal antibody**.**

FIGURE 22

Restriction digestion of pET 32 and recombinant TA vector with Kpn 1 and Sac 1 restriction enzymes

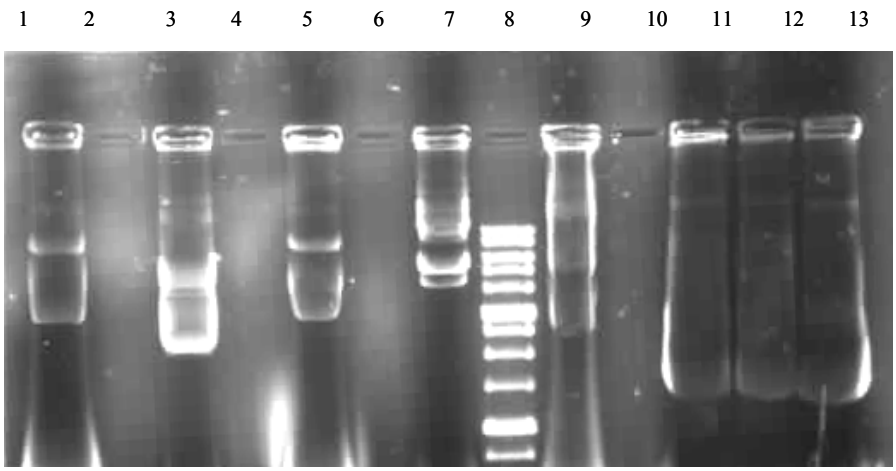


FIGURE 23

PCR analysis of transformed colonies with M 13 primers

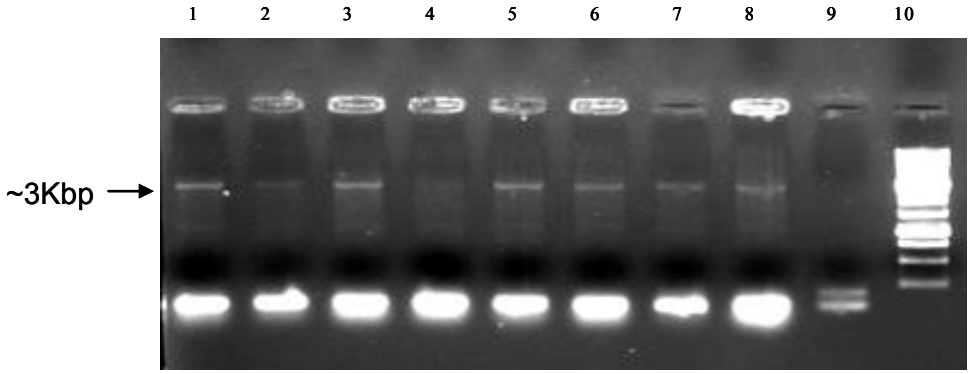


FIGURE 24

Recombinant pET32 HTa and recombinant TA plasmid DNA isolated by alkaline – SDS lysis method.

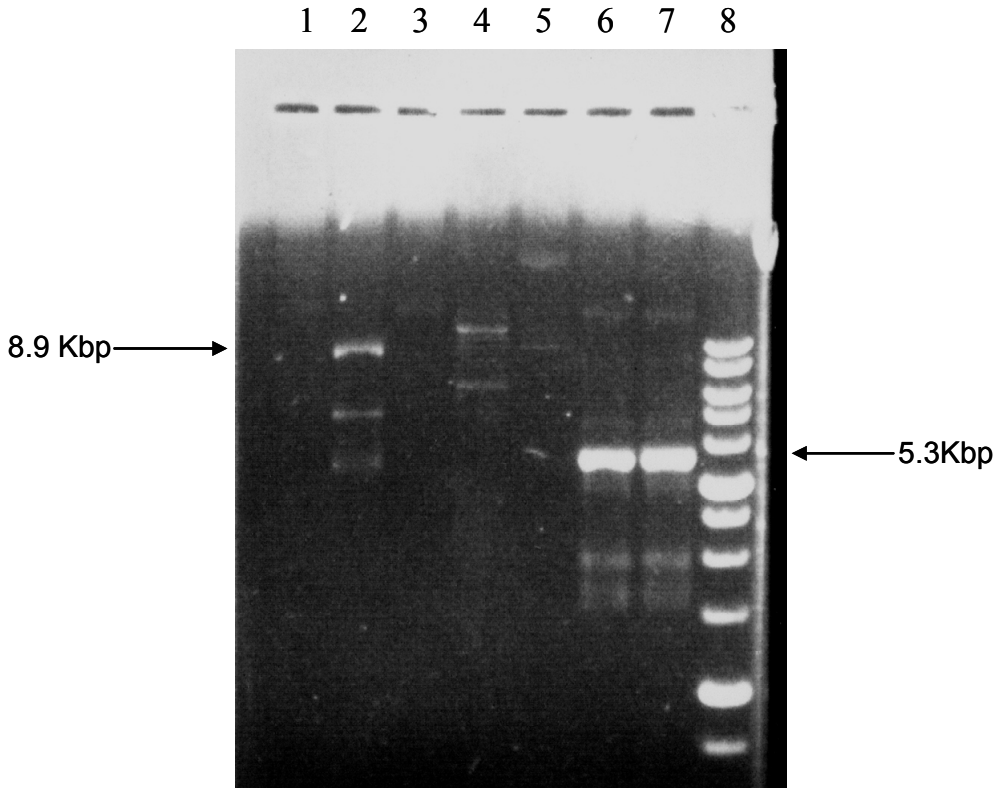
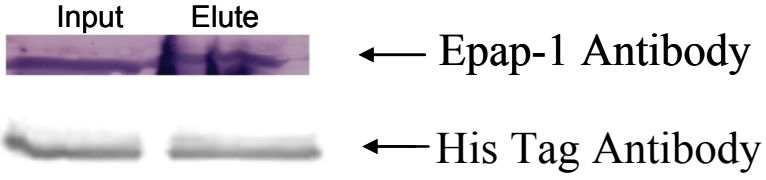


FIGURE 25

Panel A: Expression of Epap-1 in *E.coli* BL-21 cells harboring recombinant pET32 vector.

Panel B: Western Blot Analysis with Monoclonal His Tag and polyclonal Epap-1 antibody.



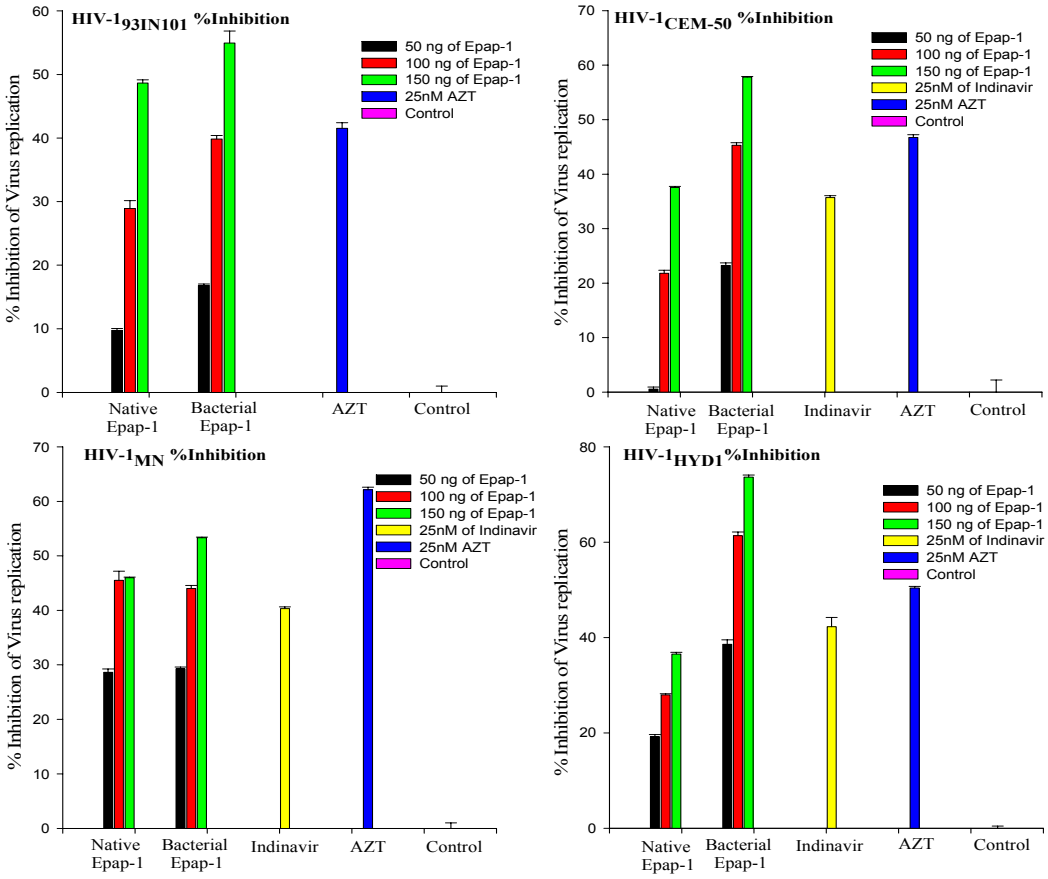
6.2. Analysis of antiviral activity of bacterial recombinant Epap-1

6.2.1. Anti-viral properties of Epap-1:

The antiviral properties of native and recombinant Epap-1 were analyzed using four HIV-1 isolates in PHA-stimulated PBMCs. The results **(Fig26)** show that Epap-1 can significantly inhibit the replication of the four HIV-1 isolates 93IN101, CEM50, HYD1 and MN suggesting that the recombinant protein can efficiently inhibit HIV-1 replication. Native as well as recombinant Epap-1 were non-cytotoxic to the cells (data not shown). The dynamics of virus replication in presence as well as absence of Epap-1 was studied by conducting the virus infection in presence of native and recombinant Epap-1 (150 ng/ml), and AZT (10 μM). The amount of virus replicated was monitored for 20 days. The results presented in **Fig 26** show that the replication of virus in the presence of native and recombinant Epap-1 is significantly inhibited, while recombinant Epap-1 also shows some variations in viral dynamics from the tenth day of infection similar to that observed in the presence of AZT. These results suggest that both native and recombinant Epap-1 preparations have significant inhibiting activity against HIV-1 replication, though the native Epap-1 has higher potential for controlling the dynamics of replication of viral sub populations.

FIGURE 26

Analysis of broad spectrum anti-viral activity of recombinant Epap-1 in PBMCs and SupT1 cells



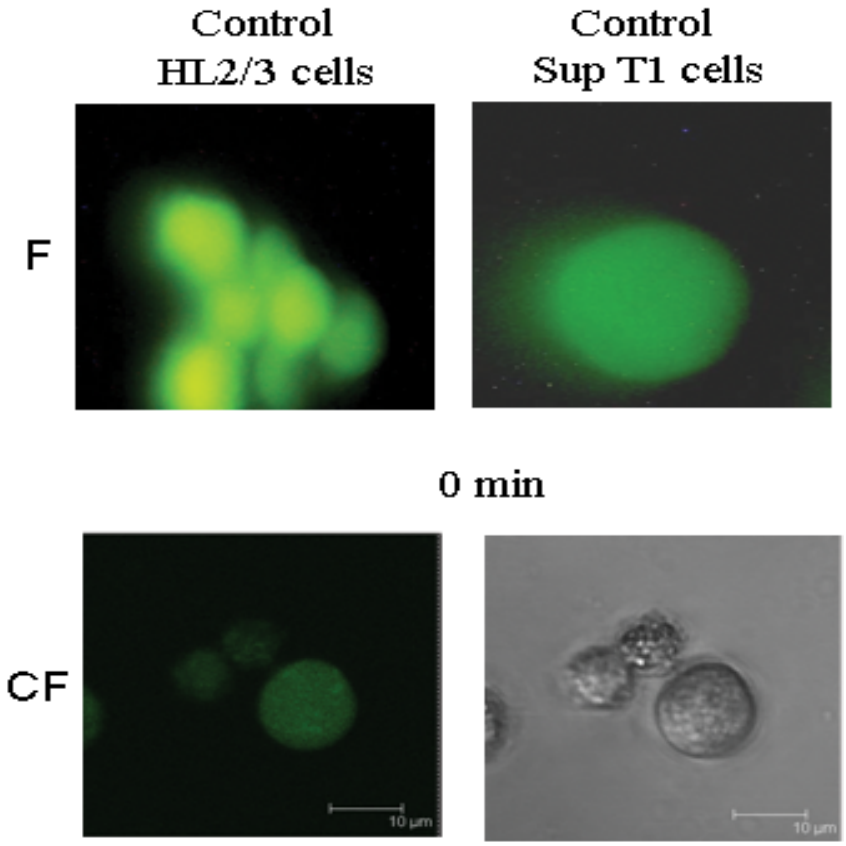
6.2.2. Action of Native and recombinant Epap-1 on gp120 mediated viral entry:

HIV-1 interaction with the sensitive host cell is dependent on the recognition of virus surface gp120 to the host cell receptors CD4 and coreceptor, followed by fusion of the virus, endocytosis and release of viral capsid. The fusion reaction of virus can be stimulated *in vitro* through fusion of a gp120 bearing cell (HL2/3) with the receptors bearing T cells (Sup T1). HL2/3 cells were loaded with Calcein AM and Sup T1 cells were loaded with Calcein blue. The green fluorescent Calcein AM in HL2/3 cells get transferred to Sup-T1 cells during fusion reaction. The ability of Epap-1 to inhibit the fusion and the dye transfer was monitored using both fluorescent and confocal microscopes. The results **(Figure 27)** show that native as well as recombinant Epap-1 can completely block gp120 mediated fusion reaction suggesting that Epap-1 inhibits viral replication through blocking gp120 mediated viral entry.

FIGURE 27

Effect of Epap-1 on viral entry: Cell fusion-Dye transfer assay

Panel A



F- Fluorescence Microscopy
CF- Confocal Microscopy

FIGURE 27

Panel B

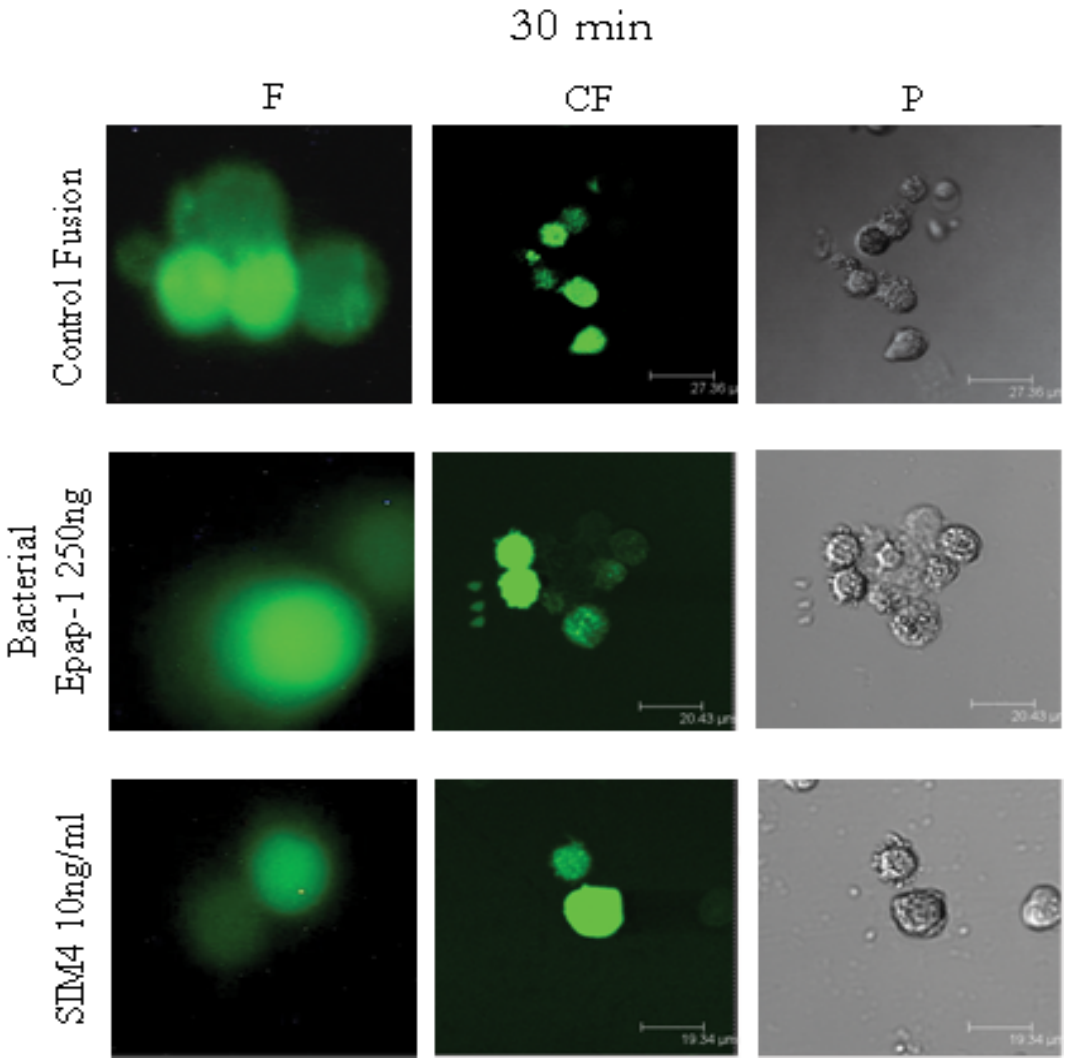


FIGURE 27

Panel C

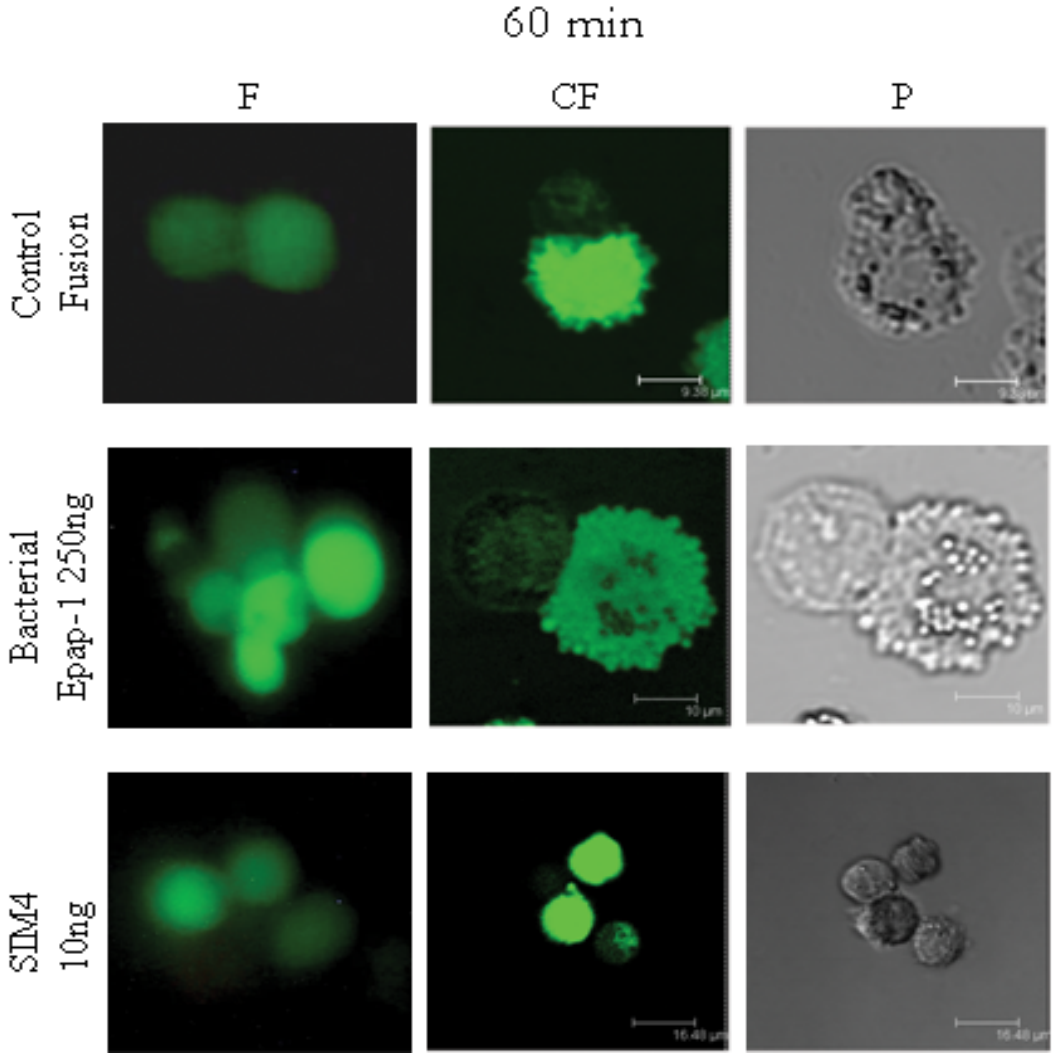
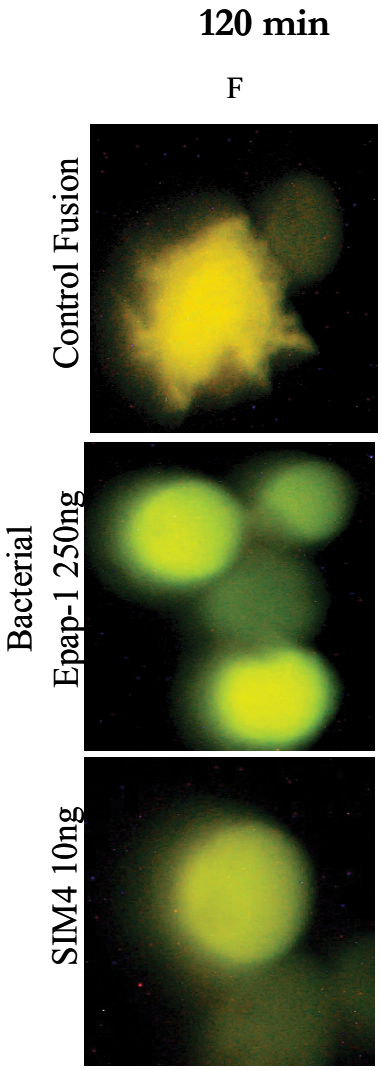


FIGURE 27

Panel D



6.2.3. Localization of Epap-1 and mapping the epitopes of HIV-1 gp120 involved in interaction with Epap-1

The analysis of Epap-1 localization in the fusion reaction using Rodamine Isothiocyanite (RITC) labeled Epap-1 shows that Epap-1 localizes on the surface of gp120 expressing HL2/3 cells **(Fig 28 Panel A)** and blocks interaction of HL2/3 cells with Sup T-1 cells. This confirms that Epap-1 blocks gp120 mediated fusion through its strong binding to the gp120 expressed on the surface of HL2/3 cells. Further analysis of RITC- Epap-1 binding in the presence of soluble CD4 show that CD4 binding could not effect (red fluorescence) the RITC-Epap-1 binding (**Fig 28 Panel B**), where as soluble gp120 can complex and reduced (red fluorescence) the RITC-Epap-1 binding (**Fig 28 Panel C**) suggesting that soluble gp120 can complex the Epap-1 and inhibit the Epap-1 binding (**Fig 28 Panel C**). This is further confirmed by the observations that gp120 blocking by whole gp120 antibody can decrease (red fluorescence) Epap-1 binding (**Fig 28 Panel D**). In addition, native as well as recombinant protein shows similar mode of action.

FIGURE 28

Panel A

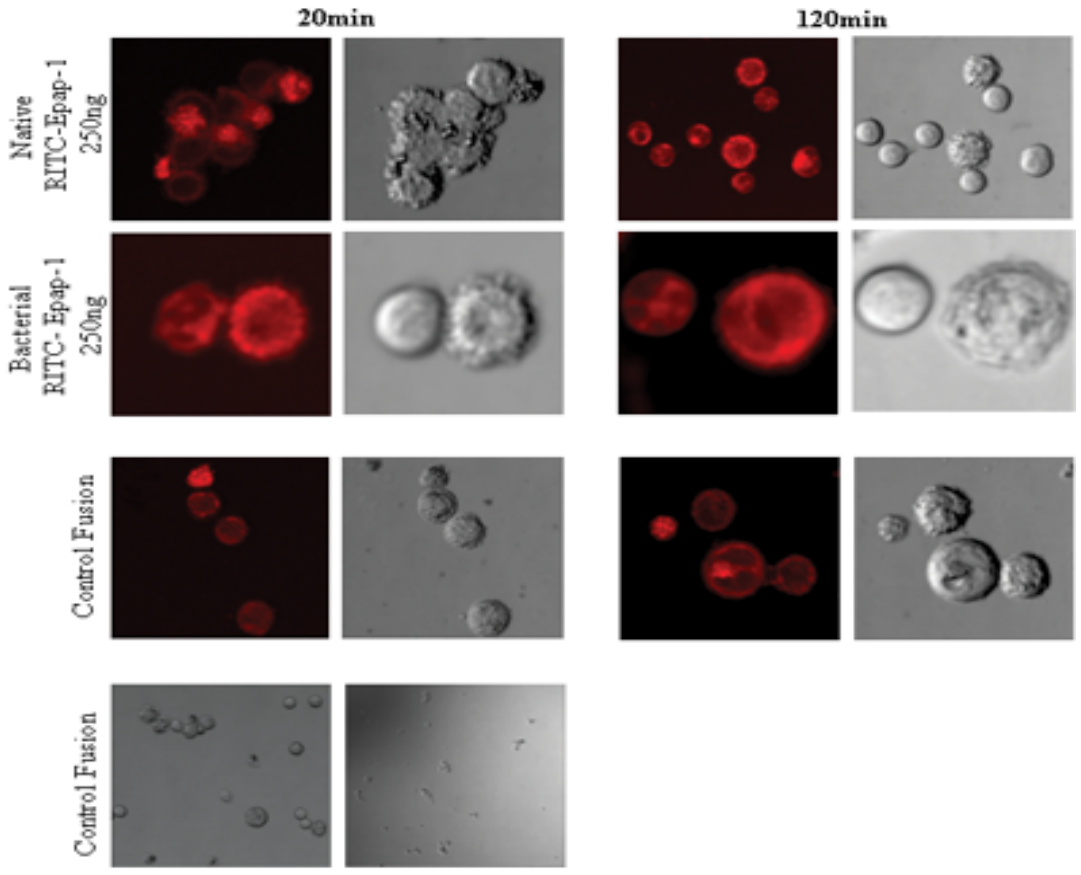


FIGURE 28

Panel B

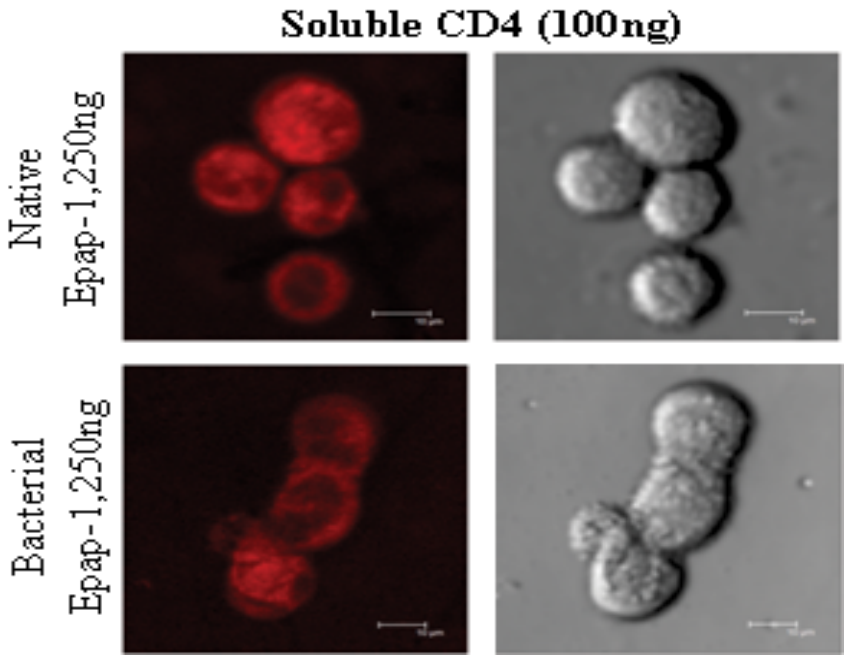


FIGURE 28

Panel C

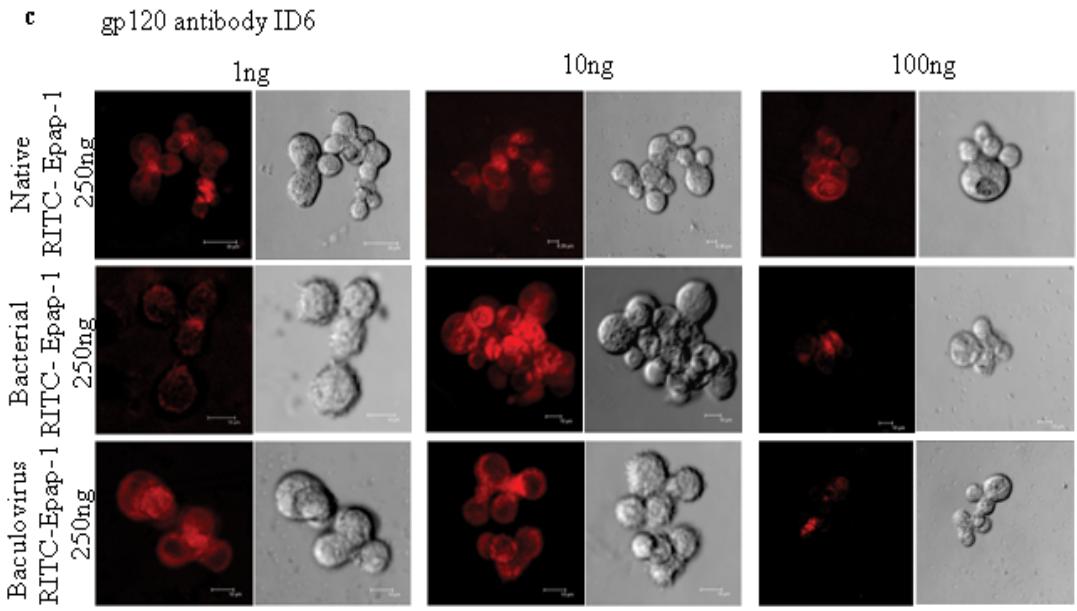
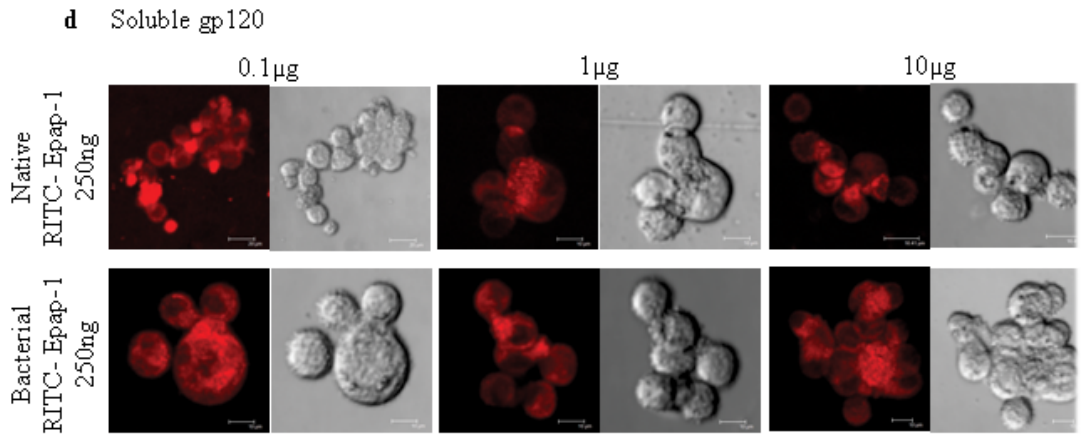


FIGURE 28

Panel D



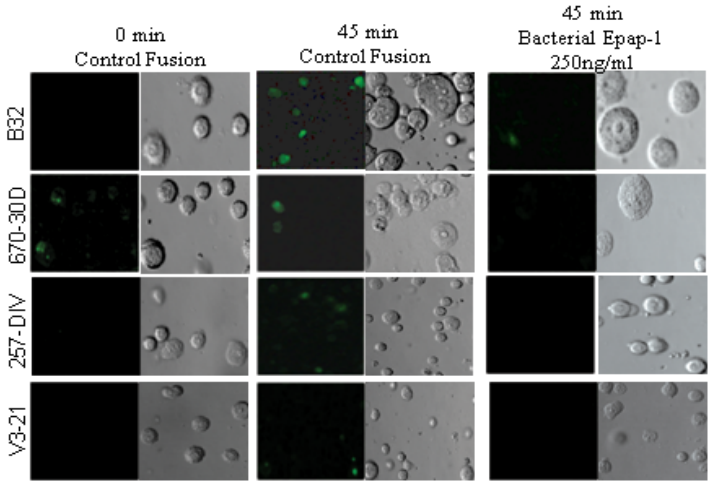
6.2.4. Analysis of envelope epitope interaction of recombinant Epap-1:

To further investigate whether such a binding of Epap-1 to HL2/3 cells is specific to certain epitopes of gp120 and whether the action of native and recombinant is conserved, we have conducted fusion reaction in the presence of Epap-1 and monitored the exposure of various epitopes of gp120 (in terms of green fluorescence) of HL2/3 cells using gp120 specific monoclonal antibodies B32, 670-30D, 257-DIV and V3-21. The results **(Fig 29 Panel A)** show that B32 epitope is highly exposed during control fusion reaction, while 670-30D, 257-DIV and V3-21 are marginally exposed. When fusion reaction was conducted in the presence of anti-human CD4 antibody (SIM4), the gp120 epitope 670-30D is completely masked, while a high exposure of B32, 257-DIV and V3-21 is observed. Where as in the fusion reaction conducted in the presence of Epap-1, B32 and V3-21 epitopes were least exposed, both 670-30D and 257-DIV were completely blocked. These results clearly demonstrate that Epap-1 binding is very specific and its recognition involved both 257-DIV and 670-30D epitopes of gp120. These findings unequivocally show that Epap-1 can block gp120 epitopes that occur in cell-mediated interaction of gp120 to T cells. The interaction of Epap-1 with 257-DIV and 670-30D may modify the conformation of gp120 and partially affect other epitopes like B32 and V3-21.

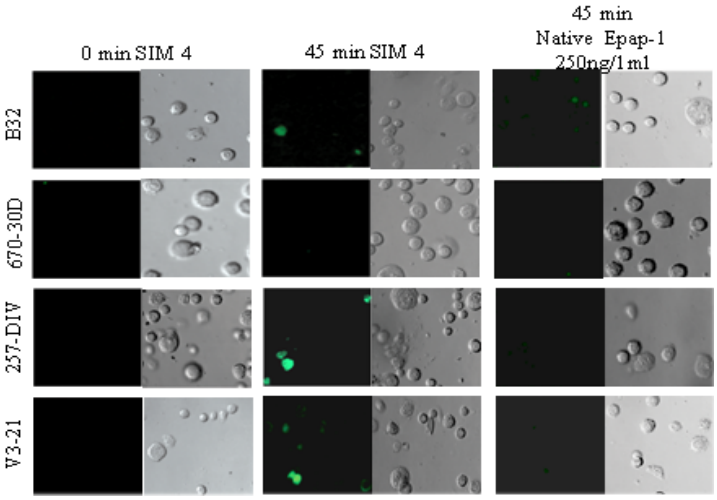
FIGURE 29

Assays for Epitope exposure pattern of HIV-1 gp120 during cell fusion reaction

Panel A



Panel B



6.2.5. Molecular interaction of Epap-1 with the cell-free virus:

Since cell free virus invades the susceptible cells in circulation in the placenta, it would be interesting to know if Epap-1 can interact with the epitopes of gp120 of cell free-virus and whether such interaction is conserved amongst the four HIV-1 isolates (as indicated). This was done by incubation of virus with increasing concentrations of Epap-1. Treated virus was captured by 3T3 CD4 cells; the epitopes of gp120 exposed in the captured virus in the presence of Epap-1 were monitored. The results **(Fig 30 Panel B)** show that 670-30D epitope of gp120 is masked in a dose dependent manner by native and recombinant Epap-1 suggesting a strong interaction of Epap-1 with 670-30D epitope present in C 5 region is highly conserved among the HIV-1 isolates 93IN101, CEM50, HYD1 and MN. This suggests a common mode of action of Epap-1 against different strains of HIV-1. Further, the involvement of the same epitopes in Epap-1 interaction with HL2/3 cells strongly indicates the conserved mode of action of Epap-1 against virus-surface and cell-surface gp120.

Host responses against pathogens are very diverse and target specific. The protection mechanisms include host genetic resistance (Farouhar et al, 2003) and innate immunity (Hilleman et al, 2004). Innate immunity is highly active in women during pregnancy. It protects the fetus against various pathogens in circulation (Madani et al, 2004). High levels of antiviral factors in HIV infected pregnant women such as neutralizing antibodies (Tranchat et al., 1999), proteins (Kondapi et al, 2002; Sharma et al, 1998), Leukemia inhibitory factor (Patterson et al, 2001), defensin peptides (Ganz et al, 1994), cytokines (Lapidotet al,2002) and CD8 cell antiviral activity (Levy, 1998) were well documented. Understanding molecular action of natural anti-viral factors will provide insight into the complex mode of action that the host adopts against pathogenic HIV. The molecular analysis of the natural antiviral protein, Epap-1, the study of its mode of action on HIV-1 infection showed that it is strongly

associated with gp120 at its principal neutralizing domain V3 loop epitope V3-F425, an epitope that potentially neutralized primary isolates (Cavacini et al, 2002). Further, the interaction of Epap-1 is so specific that it could freeze the interacting gp120-coreceptor cells without completely stopping CD4 interaction of gp120. This could be due to Epap-1 induced conformational changes in V2, C1, C3 and C5 regions that partially allowed CD4 binding, thus suggesting the requirement of multiple action of an inhibitor in bringing the virus entry to a halt. The multifaceted capability of Epap-1 action can be seen through its ability in interacting with a wide spectrum of epitopes present in soluble gp160, virus, virus-CD4 complex and virus-CD4-coreceptor complex. Since such intermediate conformations of gp120 are implicated both in cell-free virus and cell associated virus infections (Foda et al, 2001), Epap-1 possesses ability in affecting invasion of virus in both the modes. Further, the results demonstrate that the glycosylation of Epap-1 is not essential for its anti-viral activity. Thus suggesting the importance of peptide component in interaction with gp120 and inhibition of viral entry.

FIGURE 30

Mapping the epitopes of HIV-1 gp120 involved in the interaction with Epap-1using cell based ELISA

Panel A

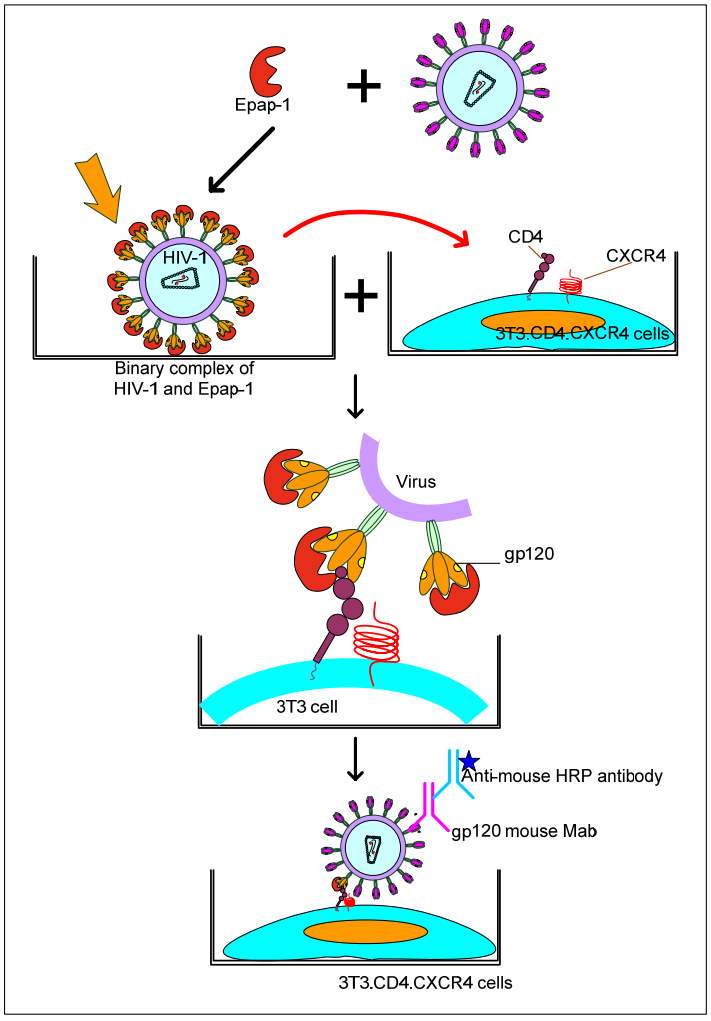
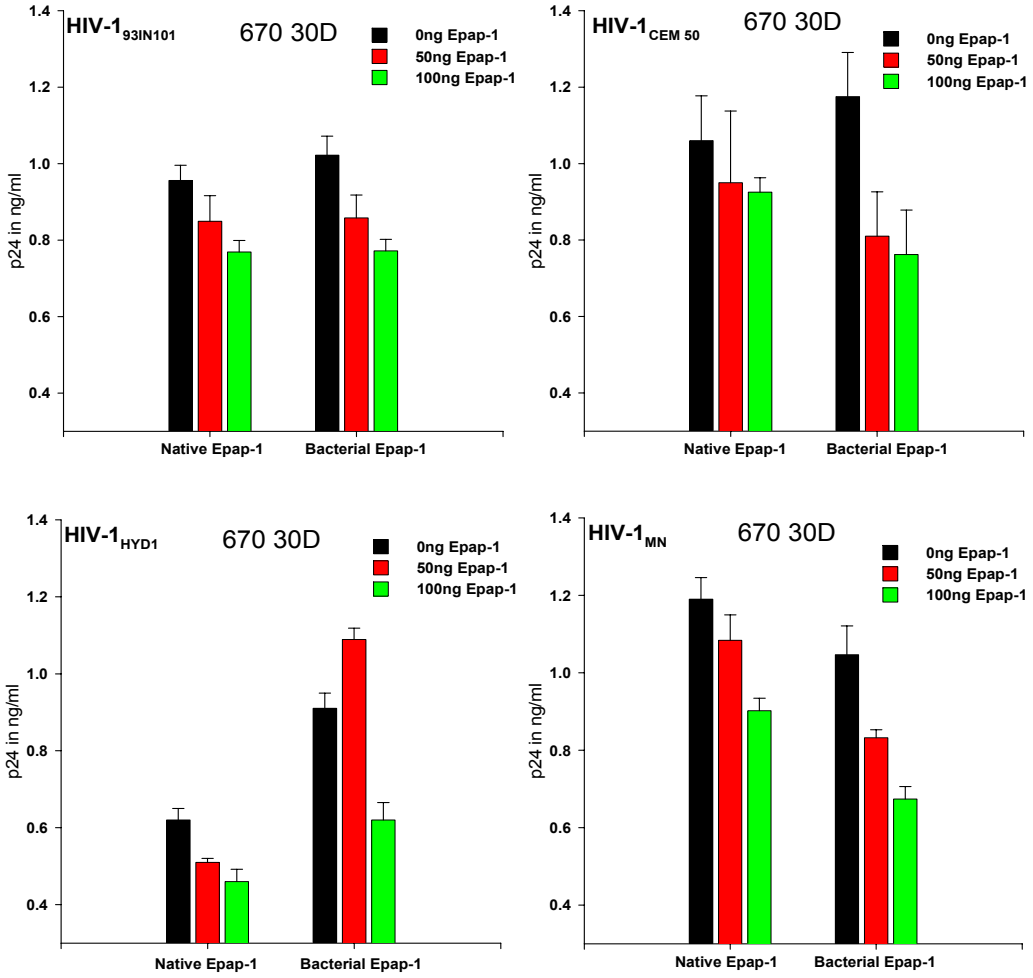


FIGURE 30

Mapping the epitopes of HIV-1 gp120 involved in the interaction with Epap-1 using cell based ELISA

Panel B



7. Conclusions

- Epap-1 secreting cells were isolated from placental villous tissue. These single nuclei cells are continuously growing as monolayer and multilayer for the past 24 months

- These cells secrete Epap-1 in cell culture supernatant which is purified to homogeneity by using SNA affinity chromatography

- The cells were vimentin positive, CD9 positive and cytokeratin negative and hCG positive. They were expressing Epap-1 and these cells grow as mono/multi layered clear and transparent cells with extended morphology with doubling time of 4 days.

- The antiviral properties of PESE cells were analyzed. The results show that endogenously expressed Epap-1 can protect PESE cells from cell free HIV infection

- The Bacterial recombinant Epap-1 was expressed in pET 32 in *E.coli* BL 21 cells and was purified using Ni Affinity chromatography.

- The bacterial recombinant Epap-1 shows anti-HIV activity and exhibits a similar mechanism of action of that of the native Epap-1. Hence, glycosylation of Epap-1 is not essential for its anti-viral activity

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9. Publications and Presentations

- PATENT: Anand K. Kondapi, University of Hyderabad & Secretary, Department of Biotechnology, Anti-HIV active bacterial and baculovirus recombinant Epap-1, Indian patent and PCT filed in December 2005.

  Patent Application # 3477 / DEL/2005, Date 26.12.2005

- Roda Rani K.P, **Dheeraj Pelluru**, Anand K. Kondapi, "A conserved molecular action of Native and recombinant Epap-1 in inhibition of HIV-1 gp120 mediated viral entry". Communicated.

- **Dheeraj Pelluru**, Anand Kumar Kondapi, Oral Presentation "Early pregnancy-associated protein-1 (Epap-1): An active anti-HIV-1 protein". Abstract presented at National Conference on Research in HIV & AIDS, New Delhi, INDIA. Slides are web casted. Dated April 21-23, 2006. Organized by NACO, India.

- **Dheeraj Pelluru**, Upendhar Gandapu and Anand K. Kondapi, "Isolation and characterization of Epap-1 secreting endothelial cells from human placental tissue: An alternate barrier for mateno-fetal transmission of virus". Manuscript under preparation.