Caveolin-1 immuno-expression in human fetal tissues during mid and late gestation

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Caveolin-1 (Cav-1) is the main protein in caveolae, and serves as a scaffolding protein onto which many classes of signalling molecules are assembled. Through interaction with proto-oncogene products, Cav-1 may suppress cell proliferation; or when phosphorylated, may also stimulate cell growth. The aim of this study was to determine Cav-1 expression in human fetal tissues, tissues composed of cells undergoing growth and differentiation processes which require a nurturing environment provided by transmembrane vesicular transport. By using immunohistochemistry, Cav-1 was detected in several fetal tissues during mid- and late gestation (from 14 to 39 weeks). The protein was present in adipocytes, endothelial cells, smooth muscle fibers and in a number of sites with a pattern of distribution similar to that of the adult. Intriguingly, a positive immunoreaction for Cav-1 was also noticed in tissues, such as the urothelium, which normally do not express this protein in adulthood. This unexpected pattern of Cav-1 in human fetus may predict novel roles for Cav-1 during fetal development.

Key words: caveolin, fetal tissues.

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Caveolae are 50-100 nm flask-shaped invaginations of plasmalemma, first detected in endothelial cells and gall-bladder epithelium by electron microscopy (Palade, 1953; Yamada, 1955). A 22-KDa protein, known as caveolin, was the initial biochemical marker for caveolae (Rothberg *et al.,* 1992). Three forms of this protein, encoded by three different and separate genes, have been heretofore described (Tang *et al.,* 1996) and denoted as caveolins 1, 2 and 3. Caveolin-1 (Cav-1) seems to be the most important of the caveolins; its two isoforms derive from the use of alternative start sequences. The full length form is the α isoform whereas the ß isoform is the shorter translation product (Scherer *et al.,* 1995). Cav-1 is essential for caveolar formation; in fact, there is evidence that Cav-1 knockout mice completely lack caveolae in non-muscle cells (Drab *et al.,* 2001; Razani *et al.,* 2001). Cav-2 is dependent on Cav-1 for caveolar localization; indeed, it is retained in the Golgi apparatus in the absence of Cav-1 (Li *et al.,* 1998; Parolini *et al.,* 1999). Cav-2 does not seem to exert a role in caveolar genesis, since Cav-2 knockout mice appear normal in terms of the presence of caveolae (Razani *et al.,* 2002). Cav-3 is mainly restricted to muscle cells (Song *et al.,* 1996).

Caveolae are thought to be involved in vesicular transport processes such as potocytosis (Anderson *et al.,* 1992), transcytosis (Bruns and Palade; 1968) and endocytosis (Schnitzer *et al.,* 1994). It has been hypothesized that extracellular substances, engulfed at caveolar regions of the plasma membrane, might be internalized by free membrane vesicles that bud from the surface and are selectively targeted to intracellular membrane compartments. Indeed, there appear to be at least three major Cav-1-containing compartments: 1) plasma membrane caveolae immobilized by cortical actin; 2) Cav-1 positive vesicles, termed *cavicles*, that move along microtubules in the cytoplasm; and 3) large pericentrosomal caveosomes (Mundy *et al.,* 2002). It has been proposed that caveolae might

also play a role in signal transduction, since these membrane structures compartmentalize a large number of components of different signalling pathways, such as the Src family tyrosine kinases, eNOS, H-Ras, epidermal growth factor receptor and G proteins (Anderson, 1993; Schnitzer *et al.,* 1994; Breton *et al.,* 1998; Oh *et al.,* 1998; Razani *et al.,* 2002). Data suggest that Cav-1 binds with these molecules within caveolar compartments and, in some instances, this binding might result in the inhibition of normal downstream signalling mediated by the sequestered protein (Li *et al.,* 1995; Li *et al.,* 1996). Subsequently, in the presence of an appropriate stimulus, Cav-1-mediated repression would be released and propagation of signalling might occur (Smart *et al.,* 1999). Thus Cav-1 is able to play an important, but controversial, role in cell proliferation; indeed it might exert tumour suppressor activity by inhibiting signalling of several proto-oncogene products following their interaction with its scaffolding domain (Razani *et al.,* 2001); nevertheless, tyrosine-14 phosphorylation of Cav-1 results in growth stimulation (Lee *et al.,* 2000).

Cav-1 is most strongly expressed in endothelial cells, adipocytes, fibroblasts and smooth muscle (Scherer *et al.,* 1997), but its presence has also been documented in other sites, including mammary gland epithelium (Lee *et al.,* 1998), lung alveolar epithelium (Newman *et al.,* 1999) and renal tubules (Breton *et al.,* 1998). Cav-1 expression has also been examined in human placenta and fetal membranes (Byrne *et al.,* 2001; Linton *et al.,* 2003). A positive immunoreaction has been found in fetal endothelial cells of chorionic villi blood vessels, villous cytotrophoblast epithelial cells and in amnionic and chorionic plate mesenchymal cells (Byrne *et al.,* 2001; Linton *et al.,* 2003). At present, no information is available on Cav-1 expression in human fetus. Fetal tissues are composed of cells undergoing intense differentiation and proliferation processes, which require a valid control system. Even more, they need a nurturing environment, provided by transmembrane vesicular transport, for their development. Cav-1 might exert a role in these mechanisms. In view of this, the aim of this study was to investigate Cav-1 distribution in fetal tissues by immunohistochemistry and to explore its eventual changes during mid- and late gestation.

Materials and Methods

Fetal tissues

Tissues from 14 fetuses of 14, 16, 19, 24, 30, 33, 36 and 39 weeks of gestation were collected in the Department of Human Pathology of S. Paolo Hospital, University of Milan, Italy, and in the Department of Human Pathology, University of Messina, Italy. Of these, the fetuses of 14, 16, 19 and 24 weeks of gestaton were retrieved following legal voluntary termination of pregnancy for maternal psychiatric disorders, according to Italian law which allows termination of pregnancy for medical reasons up to 25 weeks of gestation. In these cases, termination was induced by prostaglandin vaginal administration. Fetal death at 30, 33, 36 and 39 weeks was due to abruptio placentae for unexplained reasons and pregnancy was terminated by caesarean section, due to maternal indication. In all cases, informed consent of the mother was obtained prior to procurement of the tissues. Autopsies and sample collection were carried out immediately after delivery, assessing a less than 24h interval between fetal death and delivery. Each organ sample was formalin-fixed and paraffin-embedded for morphological diagnostic evaluation by haematoxylin/eosin staining and for immunohistochemistry. At least two samples of each fetal organ at each trimester of gestation were analyzed in at least three separate experiments.

Immunohistochemistry

Immunohistochemistry was performed on 4 μm formalin-fixed, paraffin-embedded tissue sections using a polyclonal rabbit antibody against alfa-caveolin-1 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). They were deparaffined and rehydrated through a graded series of ethanol solutions to distilled water and treated with 0.3% (v/v) H_2O_2 in absolute methanol for 30 min to quench endogenous peroxidase activity. 3, 3′-diaminobenzidine (DAB, Sigma) activated with 0.05% H^2O_2 was used as the chromogen. Sections were finally counterstained with Mayer-haematoxylin and mounted. Specificity of the binding was assessed by three kinds of controls: (i) omitting the primary antiserum, (ii) replacing it with normal rabbit serum or (iii) previously absorbing it with its homologous antigen. In addition, further controls were performed using sections taken from adult tissues

where Cav-1 has been shown to be (endothelium, smooth muscle, and adipose tissue; Scherer *et al.,* 1995), or not to be (normal urothelium; Fong *et al.,* 2003), expressed. In particular, smooth muscle, endothelium and adipose tissue present in 2 samples of adult normal large bowel wall, obtained from surgical resection, were used as positive controls, whereas urothelia of 3 adult surgically-resected ureters represented negative controls. Staining intensity was graded on a subjective scale ranging from 1 (lightly reactive) to 3 (intensely stained); 0 was given when the site stained faintly or not at all.

Results

All observations are summarized in Table 1. Immnunoreactivity for Cav-1 was evident at all considered gestational weeks within adipocytes, endothelium and smooth muscle cells (Figure 1). Positive immunostaining was also observed in adult tissues used as positive controls, while adult normal urothelium, representing a negative control, was persistently found to be negative.

In the considered fetuses, positive intense staining was observed in glandular epithelia of the digestive system organs (stomach, small- and large bowel, gallbladder). As regards the salivary glands, we found that both the serous and mucous glands expressed Cav-1.

Data concerning pancreatic parenchyma were intriguing. At the 16th week of gestation, only the exocrine component stained weakly for Cav-1. This weak staining was stable throughout intrauterine life, but from the 19th week up to term gestation (39th week), endocrine cells of the islets showed a strong immunoreaction (Figure 2).

With reference to the respiratory apparatus, laryngeal, tracheal and bronchial epithelia were not labeled until the 19th week of gestation. At this time, respiratory epithelium was still stratified and Cav-1 staining was detectable in all layers. With the progressive growth and maturation of the airways, the respiratory epithelium converts into a simple epithelium, displaying stable Cav-1 immunopositivity during mid- and late gestation.

Pneumocytes displayed a barely detectable immunoreaction at the 19th week of gestation, whereas a strong reaction was observed from the 33rd week (Figure 3).

In the urinary system, renal tubules and parietal cells of Bowman's capsule were found to stain positive at all considered gestational weeks; reaction was strong (3+) in distal tubules and only weakly detectable (1+), or absent, in proximal and collecting ones (Figure 4). A grade-2 intense positive immunoreaction was constantly noticed in bladder and ureter urothelium (Figure 5). Organs of the genital system of two female fetuses, at the 36th and 39th week of gestation respectively, were evaluated. Grade-2 intense labeling was evident in endometrium and tubal epithelium and grade-3 labeling was found in ovocytes, whereas granulosa cells stained only weakly positive.

In non-keratinized pavement stratified epithelia of the analyzed organs (esophagus, vagina, tongue), the basal layer displayed a strong immunopositivity, whereas the superficial layers were weakly positive or completely negative for Cav-1.

The epidermis showed a peculiar pattern of Cav-1 staining. At mid-gestation (16-19 weeks of gestation), a strong reaction was evident only in the basal layer (Figure 6), but with growth and progressive keratinization, the superficial layers, and especially the stratum corneum, were also Cav-1 positive.

Discussion

The present study demonstrates for the first time that Cav-1 protein is expressed in a number of human fetal tissues, at least at mid- and late gestation, a period of active tissue differentiation, growth and maturation. As expected, Cav-1 was strongly present in adipocytes, endothelium and smooth muscle cells at all gestational weeks. In addition, this protein was detected in intestinal epithelial cells, distal renal tubules and Bowman's capsule parietal cells, just as in adulthood (Field *et al.,* 1998; Breton *et al.,* 1998), where it has been postulated that it might play a role in cholesterol trafficking (Field *et al.,* 1998) and epithelial transport pathways (Breton *et al.,* 1998). Its expression in the fetus as early as the 14th week of gestation suggests a precocious role of this molecule in these processes.

Surprisingly, whereas benign human adult urothelium has been found to be negative for Cav-1 (Fong *et al.,* 2003), we found that fetal urothelium stained positive for this protein at all considered gestational weeks. Since a strong immunoreaction for Cav-1, correlated with tumor grade, has been found in urothelial carcinoma cells (Fong *et al.,*

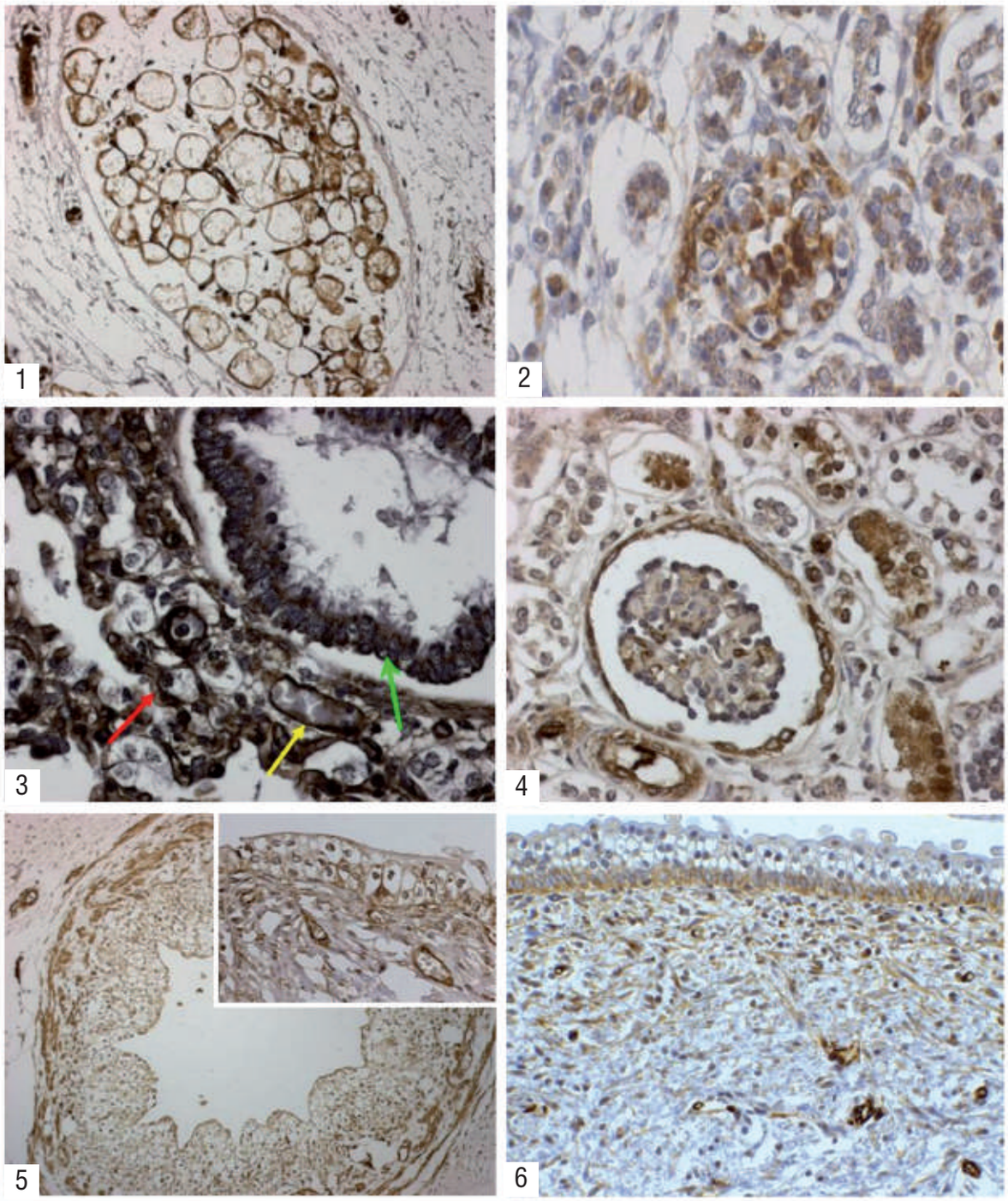

Figure 1. Cav-1 intensely positive immunoreaction in adipocytes from a fetus at 36 weeks of gestation (original magnification, x200).
Figure 2. Pancreas from a fetus at 19 weeks of gestation: inner cells of endocrine insulae are labelled by Cav-1 antiserum. A positive immunoreaction is also detectable in exocrine glands. (original magnification, x200).
Figure 3. Lung parenchyma in a fetus at 33 weeks of gestation: an intense immunoreaction for Cav-1 is evident in pneumocytes (red arrow), cells of the bronchial epithelium(green arrow) and vascular endothelial cells (yellow arrow). (original magnification, x400).
Figure 4. Renal parenchyma in a fetus at 36 weeks of gestation: Bowman's capsule parietal cells and distal tubules display a positive immunoreaction for Cav-1. Immunostaining is also evident in endothelial cells of mesangium. (original magnification, x400).
Figure 5. Ureter in a fetus at 19 weeks of gestation: urothelium and muscolaris propria are Cav-1-positive. (original magnification, x100). Inset: Cav-1-positive immuno-reaction is evident in the bladder urothelium in a fetus at 33 weeks of gestation (original magnification, x200).
Figure 6. Epidermis in a fetus at 16 weeks of gestation: basal layer is strongly labelled by Cav-1 antiserum (original magnification, x200).

Table 1. Cav-1 staining intensity, graded from 0 to 3, in different fetal tissues during pregnancy.

		WEEKS OF GESTATION						
	14 weeks	*16 weeks*	*19 weeks*	*24 weeks*	*30 weeks*	*33 weeks*	*36 weeks*	*39 weeks*
Number of fetuses	*1*	*2*	*2*	*2*	*2*	*2*	*1*	*2*
HISTOLOGICAL SITES								
Adipose tissue	3	3	3	3	3	3	3	3
Endothelium	3	3	3	3	3	3	3	3
Smooth muscle	3	3	3	3	3	3	3	3
Glandular epithelia of digestive system	3	3	3	3	3	3	3	
Salivary glands	2	2	2	2	2	2	2	2
Pancreas								
Endocrine (insulae)	Not evaluated	0	3	3	3	3	3	3
Exocrine		1	1	1	1	1	1	1
Respiratory apparatus								
Epithelia	0	0	3	3	3	3	3	3
Pneumocytes	0	0	1	1	1	3	3	3
Urinary system								
Bow man's capsule	3	3	3	3	3	3	3	3
Proximal tubules	1	1	1	1	1	1	1	1
Distal tubules	3	3	3	3	3	3	3	3
Collecting tubules	1	1	1	1	1	1	1	1
Urothelium	2	2	2	2	2	2	2	2
Female Genital apparatus								
Glandular epithelia								
Ovocytes	-	-	-	-	-	-	-	-
Granulosa cells								
Non keratinized pavement stratified epithelia	Not evaluated	3 (basal layer) 1 (superficial layers)	3 (basal layer) 1 (superficial layers)	3 (basal layer) 1 (superficial layers)	3 (basal layer) 0 (superficial layers)	3 (basal layer) 0 (superficial layers)	3 (basal layer) 1 (superficial layers)	3 (basal layer) 0 (superficial layers)
Epidermis	Not evaluated	3 (basal layer) 0 (superficial layers)	3 (basal layer) 0 (superficial layers)	3 (basal layer) 0 (superficial layers)	3 (basal layer) 1 (superficial layers)	3 (basal layer) 3 (superficial layers)	3 (basal layer) 3 (superficial layers)	3 (basal layer) 3 (superficial layers)

2003), it is tempting to speculate that Cav-1 might serve in the fetus as a promoter of mitotic activity and growth of the urinary tract.

In the present study, a peculiar pattern of Cav-1 expression was also encountered in all considered epithelial tissues, except, as already seen, in those of the gastrointestinal apparatus. We observed a different behaviour of keratinizing pavement stratified epithelia when compared with the non-keratinizing ones. Our data concerning epidermis are in agreement with the study of Sando *et al,*. (2003) regarding Cav-1 expression in human keratinocytes.

These authors speculated that, since Cav-1 is expressed in basal and corneum stratum, it might have a role in driving the formation of lamellar granules, which deliver the precursor of the stratum corneum lipids in the extracellular compartment. Nevertheless, the finding of Cav-1 expression also in basal cells of the non keratinizing pavement stratified epithelia led us to hypothesize that this protein might also serve as a factor regulating cell division processes in the highly proliferating basal cells of both epidermis and other analyzed stratified epithelia.

This hypothesis is in coherence with the studies performed by Feng *et al.,* (2002) and Kogo *et al.,* (2000), which demonstrated a key-role for Cav-1 in cytokinetic processes. These authors, indeed, found that Cav-1 is highly concentrated in the mitotic furrow during cytokinesis, and that inhibition of its activity leads to an abnormal cytokinetic process.

Cav-1 expression in adult human alveolar pneumocytes, and in bronchial respiratory epithelium has been reported (Gumbleton, 2001; Kato *et al.,* 2004). The density of caveolae herein suggests an important functional role in lung physiology. A long list of proposed caveolar functions pervades the literature, including a role in gas exchange, signal transduction, and endocytosis (Gumbleton *et al.,* 2001). In the fetus, we found a Cav-1 immunoreaction in alveolar pneumocytes starting from the 19th week of gestation, but not before. Most likely, from the progressive maturation of the lung during pregnancy there arises the need to create a valid blood-

air barrier that might function after birth, and Cav-1 could be implicated in macromolecule transport across it, including the clearance of endogenous protein from the airspaces. Furthermore, we propose that Cav-1 might also carry out a role in the regulation of alveolar and bronchial epithelial cell proliferation and differentiation. The appearance of this protein in these cells only after the 19th week suggests that it might promote cell differentiation and inhibit cell proliferation in these sites. In fact, the alveolar wall, which is normally organized in double layers, is disorganized and multilayered in Cav-1-deficient mice (Drab *et al.,* 2001).

Cav-1 production and secretion by pancreatic exocrine cells have already been reported (Liu *et al.,* 1999); nonetheless, our finding of Cav-1 immunopositivity in endocrine cells of pancreatic insulae was unexpected. Reaction was mostly evident in the core of insulae, in which beta cells are normally present. To the best of our knowledge, no data concerning Cav-1 expression in adult β cells exist at present, thus necessitating that Cav-1 function in fetal and adult endocrine pancreas be further investigated.

In conclusion, this paper provides evidence of a novel and unexpected pattern of Cav-1 expression in human fetal tissues that differs from the adult. This may reflect novel roles for Cav-1 in fetal differentiation and growth. Future studies should explore the mechanisms by which Cav-1 might regulate tissue differentiation and growth during fetal development and define the factors conditioning changes in its expression in fetal and postnatal life.

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