ABSTRACT

D2-40 is a novel monoclonal antibody that recognizes a 40,000 Da O-linked sialoglycoprotein podoplanin. Although its use is becoming more common, little work has been done with human foetuses. We initiated an evaluation of D2-40 antibody immunoreactivity in developing cutaneous adnexa of human fetuses at various gestational ages. Starting from a retrospective cohort of 1,098 human fetal autopsies we identified and selected a total of 48 fetuses ranging from the 12th week gestational age to term appropriate for this study. We demonstrated that the gems of the hair follicles were D2-40 negative in fetuses from the 12th to 15th week of gestation, positive in fetuses between the 16th and 20th week of gestation, negative in fetuses between the 16th and 20th week of gestation, negative in fetuses from the 21st week gestation to term. Normal adult controls were also negative. This is the first report to demonstrate intense D2-40 immunoreactivity in the gems of hair follicles of developing human skin.

Keywords: Human fetuses, skin appendages, hair follicles, D2-40; podoplanin, lymphatic system, immunohistochemistry

D2-40 is a novel monoclonal antibody against an oncofetal antigen, the M2A antigen, consisting of a 40-kDa sialoglycoprotein with an O-linked simple mucin-type carbohydrate structure. It has been suggested that this antibody specifically recognizes human podoplanin, and that podoplanin may be identical to the M2A antigen (1,2). The D2-40 antibody was originally considered to be useful for confirming a histological diagnosis of seminoma and dysgerminoma (3-5). D2-40 is also used as a selective immunohistochemical marker of lymphatic endothelium on formalin-fixed and paraffin-embedded tissue specimens (6,7). This antibody has been used to evaluate lymphatic invasion and clinical correlation of various neoplasms such as squamous cell carcinoma both in the head and neck region (8) and in the uterine cervix (9), as well as breast carcinoma (10) and kaposiform hemangioendothelioma (5,6,11). In this study, we evaluated immunoreactivity with the D2-40 antibody in developing cutaneous adnexa of human fetuses at various gestational ages.

MATERIALS AND METHODS

This retrospective cohort study involved a review of 1,098 human fetal autopsy files seen at our referral center from January 1987 to May 2008. Fetial tissue obtained after termination of pregnancy or miscarriage, after fetal death by abruptio placentae, maternal trauma, or related to placental disorders was considered suitable for the study. We excluded fetuses affected by
chromosome disorders, congenital malformations, and infections. We also excluded fetuses that had been dead for more than 6 hours. Termination of the pregnancy was performed at the request of the parents for medical or psychosocial reasons under currently existing Italian law. All parents were provided information and gave prior written informed consent to carry out an autopsy.

Autopsies were performed according to a previously described protocol (12). Five micron sections were obtained from paraffin blocks for both routine hematoxylin and eosin staining, and for immunohistochemical investigations. Antigenic unmasking was carried out using microwave cycles with each cycle from 4 to 32 minutes at 37° C. Blocking of endogenous biotin utilized a Ventana kit (Benchmark XT Ventana, Arizona, USA) featuring two reagents (Blocker A: avidin, specifically bonding endogenous biotin in tissues; Blocker B: free biotin saturating empty bonding sites on avidin molecules). D2-40 (Podoplanin; Signet, England, Europe) was diluted to 1:120 and incubated for 60 minutes at room temperature. The Streptavidin-Biotin Complex system was used for detection, and Diaminobenzidine (DAB) was used as the chromophore (13).

RESULTS

The study involved a total of 48 fetuses ranging from the 12th week gestational age to term. We found that the gems of the hair follicles were D2-40 negative in fetuses from the 12+0 to 15+6 week gestation, positive in fetuses between the 16+0 and 23+6 week of gestation, and negative in fetuses from the 24+0 week gestation to term (Fig. 1). Normal adult controls were also negative (Fig. 1).

DISCUSSION

This is the first report to demonstrate intense D2-40 immunoreactivity in the gems of the hair follicles of developing human skin. The intense D2-40 immunoreactivity in 16-20 gestational week fetuses, and no immunoreactivity in younger, older, or in hair gem follicles of adults may suggest an important involvement of the lymphatic system in skin development.

The complex processes of skin development have recently been extensively reviewed (14-16). Skin appendages such as teeth, hair, feathers and a number of glands including mammary glands are derivatives of the embryonic ectoderm. Although fully formed ectodermal organs differ greatly with regards to number, shape, function, and regenerative ability, the early steps in their development are remarkably similar both at the morphogenetic and molecular levels. The formation of skin appendages is regulated by reciprocal and sequential interactions between the ectodermal epithelium and the mesenchyme that can originate either from mesoderm (hair and mammary gland) or neural crest (tooth, vibrissae, and cranial hair). Early signalling steps in the specification of embryonic skin can be summarized as follows: Wnt signalling blocks the ability of early ectodermal progenitor cells to respond to FGFs, allowing them to respond to BMP signaling and to adopt an epidermal fate. In the absence of the Wnt signaling pathway, ectodermal progenitors respond to fibroblast growth factors (FGFs), down-regulate bone morphogenetic protein (BMP) signaling and progress towards neurogenesis. Some cells fail to respond to Wnts, and these become fated to become epidermal cells through BMP, Epidermal Growth Factor (EGF) and the Notch signaling pathway. The cells that do respond to Wnt signaling also receive underlying FGF and BMP inhibitory signals from the mesenchyme and, altogether, these signals instruct the cells to make an appendage. Inhibition of BMP inhibitory signals and Wnt activating signals produce the hair placode. Additional dermal messages instruct the placodes to make the follicle. We found no reports describing an involvement of the lymphatics in skin development (14-16).
It is well known that a great number of diseases that present with lymphedema as an important sign also present with various skin defects. One of the best known examples is certainly Turner syndrome. Cutis verticis gyrata, alopecia areata, vitiligo, low-set hairline, luxuriant hair growth on the arms and legs, hypertricosis, increased number of melanocytic nevi, abnormalities in tooth development are just some of the dermatological defects associated with this lymphedema-related syndrome (17).

Nail changes including slow growth, yellow-green discoloration, transverse and longitudinal overcurvature, onycholysis, shedding, cross-ridging, and loss of lunulae and cuticles are well known skin appendage defects in the lymphedema-related Yellow Nail syndrome (18) (these are only examples; an OMIM search for lymphedema and skin defects results in 33 entries). The possible relationship between the disruption sequence initiated by lymph vessel dysplasia and thereafter involving the brain and skin has been hypothesized (19). As the paper by Fuchs elegantly describes, in the absence of Wnt signaling, ectodermal progenitors respond to FGFs, down-regulate BMP signaling and progress towards neurogenesis. Demonstration of intense D2-40 immunohistochemical staining only in the buds (proliferation areas) (arrow) of the hair follicle between the 16<sup>+6</sup> and the 23<sup>+6</sup> weeks of gestation. All other gestational stages and the adult control were negative (arrows).

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**Fig. 1. Hair follicle, Hematoxylin & Eosin, and D2-40 staining in human fetuses at gestational stages: 12<sup>+0</sup> / 15<sup>+6</sup> weeks (17 cases, magnification 20x); 16<sup>+0</sup> / 23<sup>+6</sup> weeks (25 cases, magnification 20x); 24<sup>+0</sup> / 41<sup>+6</sup> weeks, (6 cases, magnification 10x); and adult control (4 cases, magnification 10x). Hematoxylin & Eosin staining (top row) demonstrated: two layers with large squamous cells constituting the periderm and cuboidal cells making up the surface of the integument at 12<sup>+0</sup> / 15<sup>+6</sup> weeks; an intermediate layer of polygonal, glycogen-rich cells is evident between the periderm and centrifugally proliferated cylindrical cells that gave origin to the fetal hair follicle at 16<sup>+0</sup> / 23<sup>+6</sup> weeks; the outer and inner epithelial sheath and shaft making up the hair follicle at 24<sup>+0</sup> / 41<sup>+6</sup> weeks; and the infundibulum, the dermal papilla, and sebaceous gland constituting the hair follicle in the adult. Immunohistochemical D2-40 staining (bottom row) demonstrates positive staining only in the buds (proliferation areas) (arrow) of the hair follicle between the 16<sup>+0</sup> and the 23<sup>+6</sup> weeks of gestation. All other gestational stages and the adult control were negative (arrows).**
reactivity in the germinal matrix layer of developing human cerebrum and cultured neural cells has recently been demonstrated (20). In this paper, Nakamura et al. suggest that both neural stem/progenitor cells and vascular and/or lymphatic endothelial cells share the same antigens and thus that there might be some developmental analogies between them. In conclusion, demonstration that D2-40, the selective monoclonal immunohistochemical marker of lymphatic endothelium was positive in the fetal gems of the hair follicles in 16-20 gestational week fetuses only may suggest the possible selective involvement of lymphatic system in the development of skin and skin appendages. In the future, filopodia studies on architecture and cellular functions (21) may help explain the clinical observations of brain and skin defects in lymphedema affected patients. This could possibly clarify how the genetic networks involved in skin development (especially in the early stages) may be related to the changes in cell proliferation, death, migration, adhesion and polarity which are essential for successful organ formation.

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