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Ultrastructural Characterization of Hyperactive Endothelial Cells, Pericytes and Fibroblasts in Hypertrophic and Nodular Port Wine Stain Lesions

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To the editor: Port wine stain (PWS) is a congenital vascular malformation of human skin involving the superficial vascular plexus,[1-4] but the molecular pathogenesis of these lesions remains incompletely understood.[5-8] We herein performed a transmission electron microscopy (TEM) study to determine the main pathological characteristics and ultrastructure of various cell types, including endothelial cells (ECs), pericytes, fibroblasts and keratinocytes, in hypertrophic and nodular PWS. The study was

approved by the Investigational Review Board at the Xijing Hospital, Xi'an, China. A total of 8 hypertrophic, 8 nodular PWS and 8 normal control facial specimens adjacent to the lesions were obtained and de-identified for this study.

ECs and pericytes: With TEM, normal vessels exhibited prominent ECs with minimal basement membrane thickening and pericytes abutting the basement membrane zone in the normal control skin (Figure 1a). In all hypertrophic and nodular PWS specimens, hob nailing of ECs with striking gaps between the ECs was prominent. There was lateral contact with pericytes by loose, wavy and multiple layers of basement membranes (Figures 1b-d). The main characteristics of these ECs were the presence of large numbers of rough endoplasmic reticulum (rERs), stacks of Golgi complexes, vesicles, free ribosomes and distended mitochondria in the cytoplasm (Figures 1b-d), which were observed in all subjects. Binucleated ECs were observed in 5 subjects, indicating a proliferative phase (Figure 1d). These predominant ultrastructural characteristics represent a highly metabolic cytoplasm and suggest that ECs are hyperactive in hypertrophic and nodular PWS. In normal control skin, pericytes were closely associated with ECs, showing common organelles such as rER, mitochondria and vesicles (Figure 1e). In all hypertrophic and nodular PWS specimens, larger mitochondria, denser ribosomes and a larger number of vesicles in the cytoplasm of pericytes were observed as compared to normal control skin (Figures 1f-h), indicating the very striking hyperactive state of pericytes.

Fibroblasts and collagen: In normal control skin, fibroblasts contained common organelles, such as Golgi complexes, ribosomes and rER, vesicles, mitochondria (Figure 1i). In all hypertrophic and nodular PWS specimens, many fibroblasts showed very high biosynthetic activity with a cytoplasm full of rER (Figures 1j,k) while some fibroblasts showed a hypermetabolic state containing large numbers of distended vesicles and mitochondria (Figure 1l). Binucleated fibroblasts were observed in all subjects, indicating a proliferative phase (Figure 1l). In normal control skin, small collagenous fiber bundles (1 to 5 μm in width), consisting of small-diameter fibrils, were assembled in an irregular meshwork in the papillary dermis (Figure 1m). In all hypertrophic and nodular PWS specimens, much larger collagenous bundles (5-40 and

5-150 μm in width, respectively) were packed beneath the epidermis (Figures 1n, o), which have resulted from the hyperactive fibroblasts. These fiber bundles tended to be parallel to the skin surface. Some secretion active fibroblasts were scattered among the hypertrophied collagenous fibers hypertrophic and nodular PWS. Some fibroblasts appeared to be in a degenerative state (Figures 1n, o). These data are consistent with our recent findings regarding hypertrophy and anisotropic orientations of collagen fibers in infantile and early childhood PWS skin.[9]

Epidermis and keratinocytes: In normal control skin, sparse melanosomes of various sizes were observed at the keratinocyte basal layer (Figure 2a). The tono-filamentous bundles (tonofibrils), assembled by keratin filaments, were organized in the juxtanuclear region of the cytoplasm and oriented perpendicular to the plane of the epidermal-dermal junction (Figure 2b). Cytoplasmic extension of the keratinocytes and normal epidermal-dermal junction with basal lamina and lamina lucida were prominent (Figures 2c-e). In hypertrophic and nodular PWS specimens from 5 out of 8 subjects, clustered melanosomes were more frequently observed at the keratinocyte basal layer (Figures 2f, g, k, l,). Larger diameter tonofibrils were also present in 5 out of 8 subjects in the juxtanuclear region of the cytoplasm as compared to normal control skin (Figures 2g, l). Microcytic debris, containing structural components of epidermal-dermal junctions and formed by the detachment and encapsulation of micro-portions of cytoplasmic extensions of keratinocytes, were observed in 6 out of 8 subjects in the papillary dermis (Figures 2h, m). Furthermore, in hypertrophic and nodular specimens from 4 PWS subjects, tonofibrils tended to be more clustered and intercellular spaces among the basal layer of keratinocytes were more conspicuous as compared to normal control skin (Figures 2d, i, n). The densities of individual hemidesomes were much less and the basal lamina thickness was much thinner in hypertrophic and nodular PWS specimens from 5 subjects as compared to normal control skin (Figures 2e, j, o). It was noteworthy that breakdown of the basal lamina was observed in 4 out of 8 subjects in the epidermal-dermal junctions in nodular PWS (Figure 2o). These results together suggested that epidermal-dermal junctions in hypertrophic and nodular PWS have undergone degeneration.

G alpha subunit q (GNAQ) mutation (R183Q) status: The GNAQ (R183Q) has been reported to be linked to the vascular phenotypes of PWS.[5-8] We performed a whole exome sequence on the specimens from 4 out of 8 subjects. We identified GNAQ (R183Q) in the lesions from all subjects, with mutation frequencies of 4%, 5%, 6% and 4% in hypertrophic and 14%, 2%, 4% and 7% in nodular specimens, respectively. GNAQ (R183Q) was found in the normal skin from one subject with a 4% mutation frequency, but not in the normal skins from other three subjects. The GNAQ (R183Q) status in the remaining 4 subjects is unknown and will be determined in future. Figures 1 and 2 were from the specimens with GNAQ (R183Q) confirmed.

In summary, we found that hyperactive ECs, pericytes and fibroblasts were the main characteristics present in hypertrophic and nodular PWS, which suggests their roles in the proliferation of PWS blood vessels and replication of the stroma may eventually result in the cellular phenotypes of hypertrophic and nodular PWS. We will perform a comprehensive study to quantify the changes between normal skin and hypertrophic and nodular PWS lesions in the future.

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Figure legends

Figure 1 Hyperactive ECs, pericytes and fibroblasts in hypertrophic and nodular PWS. (a) A normal capillary in normal control skin. (b) A dilated blood vessel with ECs bulging out from the basement membrane zone. The loss of EC-EC tight junctions (red arrow) and breakdown of basement membrane (blue arrows) was observed. (c) and (d) Individual ECs from two different dilated blood vessels in nodular PWS. Note a binucleated EC shown in (d). There were numerous vesicles, free ribosomes, rERs and mitochondria in the cytoplasm of these ECs. (e) A normal capillary with a pericyte abutting the basement membrane zones in normal control skin. (f) An active pericyte in a hypertrophic PWS surrounded by hypertrophied collagenous fibers showing a cytoplasm full of mitochondria, rERs, free ribosomes and vesicles. (g) A roundish hyperactive pericyte in a PWS blood vessel from nodules shows very large numbers of rERs, free ribosomes and vesicles in the cytoplasm. (h) TEM image with a higher magnification of the boxed area in (g). (i) Normal fibroblasts showing common abundant organelles in the cytoplasm, such as mitochondria, Golgi complexes, in the normal control skin. (j) An active fibroblast in a hypertrophic PWS with numerous rER in the cytoplasm. (k) and (l) Hyperactive fibroblasts in PWS nodules show a very high density of organelles, such as rERs, free ribosomes, mitochondria, and vesicles. Note the binucleated fibroblast in (l). (m) small collagenous fiber bundles present in the papillary dermis of normal control skin. (n) Hypertrophied collagenous bundles beneath the epidermis in hypertrophic PWS lesions. (o) Enlarged collagenous bundles in the dermis from PWS nodules. Active fibroblasts are scattered among the hypertrophied collagenous fibers in both hypertrophic and nodular PWS lesions (red arrows). Some fibroblasts appear to be in a degenerative state (blue arrows). The red insets represent higher magnification of the boxed areas in each image. E, endothelial cells; P, pericyte; B.V., blood vessel; G, Golgi complex; v, vesicle; m, mitochondria; r, free ribosomes; r.E.,

rough endoplasmic reticulum; C, collagen; F, fibroblast; K, keratinocyte; n, nuclear. Scale bar: 1 μm in (a)-(i) and 5 μm in (m)-(o).

Figure 2 Abnormalities of keratinocytes and epidermal basal layer in hypertrophic and nodular PWS. (a) Normal basal layer of keratinocytes show dispersed melanosomes, organized tonofibrils in the juxtanuclear region of the cytoplasm (b) and cytoplasmic extensions anchored to the papillary dermis (c) in the normal control skin. (d) Normal epidermal basal layer shows well organized alignments of keratinocytes and even distribution of tonofibrils (yellow arrows) in the intercellular spaces among keratinocytes in the normal control skin. (e) Structural components of epidermal-dermal junctions such as hemidesomes (purple arrows) and basal lamina (green arrows) are prominent. In the hypertrophic (f-g) and nodular (k-l) PWS, clustered melanosomes (red arrows) and enlarged tonofibrils are more frequently observed. (h) and (m) Detachment and encapsulation of cytoplasmic extensions of keratinocytes form microcytic debris (blue arrows) which appear in the papillary dermis. The components of epidermal-dermal junctions, such as hemidesomes, tonofibrils, lamina lucida and basal lamina were evident (indicated in the red insets). In the hypertrophic (i-j) and nodular (n-o) PWS, tonofibrils are more clustered and intercellular spaces among keratinocytes are more conspicuous (yellow arrows). The densities of individual hemidesomes are less (purple arrows). The basal lamina thickness in the epidermal-dermal junctions is much thinner (green arrow) with many breakdowns (red arrows) in nodular PWS (o). (b), (g), (l), (e), (j) and (o) are TEM images with higher magnifications of the blue boxed areas in (a), (f), (k), (d), (i) and (n), respectively. The red insets in (e), (h), (j), (m) and (o) represent higher magnification of the yellow boxed areas from each image. K, keratinocyte; Ex, cytoplasmic extension; N, nuclear; tf, tonofibrils; M, melanocyte. Scale bar: 1 μm.