Skin microvascular adaptations during maturation and aging of hairless mice

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INCREASE OF TISSUE MASS during juvenile maturation may require distinct changes of the microvasculature aiming at adequate blood flow for the enlarged tissue. In general, angiogenic processes, i.e., new vessel formation, but also remodeling and elongation of existing microvessels, are potential ways that the microvasculature may meet the needs of increasing tissue mass from juvenile to adult life (17, 18). During senescence, nutritional needs of organs might again change when the onset of functional decline occurs. Detailed information on age-related adaptations of the microvasculature is necessary for the understanding of development and aging of an organism. However, so far there are no studies dealing with changes in vascular architecture of any individual tissue during the whole life span of an animal. This is, at least in part, because of the fact that the analysis of microvascular development essentially requires the study of identical regions of tissue over time and that most of the animal models do not satisfy this requirement and do not permit quantitative analysis without surgery and invasive handling of tissue. With the present intravital fluorescence microscopic study, we introduce an experimental approach in the hairless mouse that allowed us to repetitively assess the skin microvasculature over the extended time period of an animal’s life without the need of surgical preparation. The mouse ear is accessible with minimal handling and provides a fingerprint-like angioarchitecture, making any vessel identification redundant. The major purpose was to determine both vascular adaptations of the skin during growth of mice from juvenile to adult and during aging from adulthood to senescent life, respectively.

METHODS

Animals. In conformity with the guiding principles for research involving animals, eight male homozygous (hr/hr) mice (3, 7) purchased from Charles River Laboratories (Sulzdorf, Germany) were used in this study. The animals were housed in single cages at a temperature of 22–24°C at a relative humidity of 60–65% with a 12:12-h day-night cycle. The animals were allowed free access to drinking water and standard laboratory chow (Altromin, Lage, Germany). At the first experimental time point, mice were 6 wk old. This age was selected so that the animals, although clearly immature, were well beyond weaning (~3 wk) and were free of neonatal characteristics. At 6 wk of age, the mouse ear, which consists of a single layer of cartilage sandwiched between two full dermal layers of skin (overall thickness 300 μm), is completely naked (3). Blood supply is provided by three vascular bundles, entering the ear at its base and forming an interconnecting capillary network at the periphery.

Intravital fluorescence microscopy. For the analysis of the cutaneous microcirculation of the ear, the mice were intraperitoneally anesthetized with a mixture of ketamine (90 mg/kg body wt) and xylazine (25 mg/kg body wt) and placed prone on a Plexiglas pad. After injection of 0.15 ml 5% fluorescein-isothiocyanate (FITC)-labeled dextran (molecular weight 150,000; Sigma Chemical, St. Louis, MO) via a tail
vein, the ear to be investigated was gently extended over a microscopic slide embedded into the pad and covered with an oxygen-impermeable plastic wrap. The anesthetized mice were then placed with the Plexiglas pad under an intravital Leitz Orthoplan microscope (Leitz, Wetzlar, Germany). The epi-illumination microscopic setup included a 100-W mercury lamp and a Ploemopak illuminator equipped with an I2 blue filter (450- to 490-nm excitation, >580-nm emission wavelength). Microscopic images were recorded by a charge-coupled device video camera (CP8/1FMC, Kappa, Gleichen, Germany) and recorded on videotape (Panasonic AG-7350-SVHS, Matsushita, Tokyo, Japan) for subsequent offline evaluation.

**Analysis of skin microvasculature.** The microscopic procedure for analysis of skin microvasculature was performed at a constant room temperature of 23°C. Contrast enhancement for visualization of the cutaneous vasculature with the individual microvascular segments was provided by FITC-dextran (mol wt 150,000), which binds neither to endothelial cells nor to individual blood cells and which does not extravasate but forms physiological capillaries. The dye remains for >14 h within the intravascular space and is cleared from the circulation by both the liver and the kidney. Leitz objectives [×2.5, NA (numerical aperture) = 0.08; ×10, NA = 0.3; and ×20 long distance (L), NA = 0.32] were used in the recordings and produced a total magnification of approximately ×100, ×400, and ×800 at the video monitor. With the ×2.5 objective, the extended ear was scanned in a meandering manner through the entire longitudinal and transverse lengths to monitor the whole area as well as to count the number of arterioles (first, second, third, and fourth order) and venules (first, second, third, fourth, and fifth order). At the first time point of observation (6 wk), distinct tissue regions of interest, which had an easily identifiable branching pattern of either arterioles or venules, were selected and video printouts were made during videography using the ×10 objective. Within those tissue regions, functional capillary density, i.e., the length of red blood cell-perfused capillaries per observation area (cm/cm²), was monitored and analyzed by means of the ×20 L objective. The video prints were initially marked to indicate the exact locations for measurements of length, diameter, tortuosity, and distances between the individual microvessels.

All parameters were analyzed offline with a computer-assisted image analysis system (CapImage, Zeintl Software, Heidelberg, Germany) (10), including planimetric assessment of mean area of the ×100 recordings by tracing along the boundaries of each ear section with a computerized digitizing tablet. From the ×400 recordings, functional capillary density, tortuosity of arterioles and venules, arteriolar and venular diameters, red blood cell velocity, and arteriolar and venular vessel segment length were assessed. Red blood cell velocity was determined by means of the line-shift method in the red blood cell capillaries by either the merging of venules or the branching of arterioles.

**RESULTS**

At the first experimental time point, the 6-wk-old animals had a mean body wt of 22.8 ± 0.2 g (Table 1), which significantly (P < 0.01) increased by 35 ± 5% by the age of 36 wk (Table 1). No significant weight gain or
loss was observed during the second half of the observation time period up to the age of 78 wk (Table 1). Initially, the average area of the mice ears was 39.8 ± 1.3 mm², which significantly increased by 26 ± 1% to a mean area of 50.5 ± 2.3 mm² (P < 0.01) between 6 and 36 wk of age. The 1.35 ± 0.05 times increase in body weight compared with the 1.26 ± 0.01 times increase of the area of the ears indicates that surface area of the ear does not proportionally increase as much as whole body mass during the late juvenile maturation phase and early adult life. Within the time period of 36 to 78 wk of age, the ear area was found to be slightly reduced in the range of 116–122% of the initial size (Table 1).

During growth, adulthood, and senescence, the number of both arterioles and venules as well as the number and pattern of vessel branches remained essentially constant (Table 2, Fig. 1). Moreover, the angle between branching arterioles and merging venules did not change over the time period of 6 to 78 wk of age (Fig. 1). However, within the life span from juvenile to early adult, assessment of the individual vascular segment lengths revealed an elongation of vessels. In both arteriolar and venular vessels with diameters in the range of 10–100 µm, a marked (P < 0.01) increase of the segment length between successive branching points was found with no significant difference in the extent of elongation between either arteriolar and venular or smaller and larger vessels, respectively (Figs. 2 and 6, A and B). This increase in length over time ceased at the age of ~36–42 wk (early to late adult life). In parallel, volumetric blood flow increased comparably in both arterioles and venules only from early lifetime up to the age of 36 wk (Table 3). During the subsequent life time period, volumetric blood flow in arterioles and venules remained almost constant (Table 3), but a moderate shortening of vessel segment lengths occurred, probably due to an age-related tissue shrinkage (Fig. 2).

The age-related elongation of vessels, which can be interpreted as longitudinal growth, was associated with a significant (P < 0.01) increase of the intervesselular distances, i.e., the arterioarteriolar and the venovenular distances, by ~10% within the first 36 wk of age (Figs. 3 and 6, A and B). This transverse dispersion of arteriolar and venular vessels slightly decreased during late adulthood and senescence to 78 wk of age (Fig. 3).

Regression analysis revealed significant (P < 0.01) correlations between the increase of the ear area and both the longitudinal growth, as given by the vascular segment lengths, and the transverse growth, as given by the intervesselular distances (Figs. 4 and 5).

In addition to the increase in lengths, venules with a diameter <40 µm became tortuous, with ratios of the

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### Table 1. Body weight, area, and cutaneous functional capillary density of ears of 8 hairless mice during their growth from late juvenile to adult and senescent life (6–78 wk)

<table>
<thead>
<tr>
<th>Week</th>
<th>Body wt, g</th>
<th>Ear area, mm²</th>
<th>Functional capillary density, cm²/mm²</th>
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<td>12</td>
<td>27.5 ± 0.5</td>
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<td>78</td>
<td>30.3 ± 4.8</td>
<td>31.5 ± 3.7</td>
<td>37.5 ± 1.4</td>
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</table>

Values are means ± SE. Nos. in heading indicate week. Within the mice ears, identical tissue regions were repetitively studied using intravital fluorescence microscopy for the analysis of age-related adaptations of the skin microcirculation. *P < 0.01 vs. 6 wk; †P < 0.01 vs. 36 wk.

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### Table 2. Changes in number of arterioles and venules in the exact same tissue regions of ears of 8 hairless mice during their growth from late juvenile to adult and senescent life (6–78 wk)

<table>
<thead>
<tr>
<th>Week</th>
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<th>18</th>
<th>24</th>
<th>30</th>
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Percent changes of the number of first (1A; N = 32), second (2A; N = 53), third (3A; N = 27), and fourth (4A; N = 4)-order arterioles and first (1V; N = 20), second (2V; N = 63), third (3V; N = 175), fourth (4V; N = 119), and fifth (5V; N = 34)-order venules of the skin from 6 to 78 wk of age. Vessels were compared on a one-to-one basis so that the gain column represents the maximal number of vessels and the loss column represents the minimum number of vessels seen in comparison with the number of vessels at 6 wk of age, respectively. The change column represents the net difference of vessel gain and loss. Ratios were calculated between the number of vessels at both 36 (T36) and 66 wk of age (T66) and 6 wk of age (T6).
actual path length to the straight line length increasing from 1.12 ± 0.02 at 6 wk to 1.17 ± 0.03 at 36 wk of age (P < 0.01) with no further change from late adult to senescent life (1.16 ± 0.06) (Figs. 6, C and D, and 7). In contrast, venules with diameters >40 μm did not increase in tortuosity (Figs. 6, C and D, and 7). Moreover, arterioles (diameter range: 15.0–52.5 μm) failed to exhibit a tortuous path at either of the mice ages studied (Figs. 6, C and D, and 7).

During maturation, adulthood, and aging to senescence, analysis of vessel diameters that were assessed at specific locations along the arterioles and venules showed a progressive (P < 0.01) increase in arterioles with initial diameters <20 μm, whereas larger arterioles, i.e., those with diameters >20 μm, remained in the range of 100 and 120% of their diameters at 6 wk of age. Venules of either diameter, <40 μm and >40 μm, exhibited an inconsistent pattern of dilatation up to a maximum of 120% within the life span of 6 to 78 wk (Table 4).

The quantitative assessment of the functional capillary density revealed an initial mean value of 74.5 ± 3.7/cm². During the life span from 6 to 36 wk, density of capillaries was maintained (Table 1), which, in regard to the increased ear size, implies the recruitment or new formation of microvessels. In contrast, with aging up to 78 wk, functional capillary density progressively decreased to 43.2 ± 4.3% (P < 0.01; Table 1).

As a functional parameter, reactive hyperemic response to short ischemia of the ear was tested in relation to the animals' age. By intravital fluorescence microscopy, we found an increase of arteriolar diameters during initial postischemic reperfusion, however, without any differences between the groups (Fig. 8). Of note, arteriolar blood flow velocity, which ranged between 515 and 1,180 μm/s at baseline, increased on reperfusion up to 120% of baseline values in young animals, whereas the mid-aged animals were only able to reconstitute blood flow velocity to preischemic baseline values. Strikingly, old animals failed to show a hyperemic response inasmuch as blood flow velocity remained significantly lowered during reperfusion (65 ± 9% of baseline values; Fig. 8).

Analysis of tissue PO2 revealed a slight difference in cutaneous oxygenation already at baseline conditions in that animals with higher age showed a tendency toward lower tissue PO2 values. Differences between the three groups became more evident during the initial reperfusion period: although young and mid-aged animals exhibited a marked hyperemic response, reflected by an increase of cutaneous PO2 to 202 and 193% of preischemic baseline values, hyperemic response in old animals was found limited in extent with a PO2 of only ~150% of baseline (Fig. 8).
DISCUSSION

These experiments show that neither maturation nor aging significantly modifies the overall vascular branching pattern of ear microvasculature. Microvascular dimensions simply expand in length to suit the increased dimensions of mice ears. There is no indication for age-related new vessel formation, except the fact that unchanged functional capillary density implies formation of new capillaries, because the ear dimensions expanded. Enlargement of feeding and draining vessels as well as maintenance of vascular density at the capillary level seem to be the compensatory mechanisms to match perfusion to the ear growth up to the early adult life. In addition to a substantial reduction of functional capillary density, there is no particular remodeling of the skin microvasculature in response to aging to senescent life.

### Table 3. Volumetric blood flow in arterioles and venules of ears of 8 hairless mice during their growth from late juvenile to adult and senescent life (6–78 wk)

<table>
<thead>
<tr>
<th>Week</th>
<th>Arteriolar volumetric blood flow, % of baseline</th>
<th>Venular volumetric blood flow, % of baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>100 ± 5 125 ± 7 124 ± 9 135 ± 9 146 ± 16 182 ± 11</td>
<td>100 ± 7 107 ± 27 113 ± 24 137 ± 24 140 ± 30 185 ± 35</td>
</tr>
<tr>
<td>12</td>
<td>182 ± 11 191 ± 15 192 ± 23 188 ± 16 179 ± 28 177 ± 29</td>
<td>173 ± 32 176 ± 44 172 ± 62 176 ± 34 179 ± 25</td>
</tr>
<tr>
<td>18</td>
<td>157 ± 16 146 ± 8</td>
<td>132 ± 35 79 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Nos. in heading indicate week. Within the mice ears, identical tissue regions were repetitively studied using intravital fluorescence microscopy for the analysis of age-related adaptations of the skin microcirculation. *P < 0.05, †P < 0.01 vs. 6 wk.

Fig. 3. Arterioarteriolar and venovenular distances (percentage of values at 6 wk of age) within the ears of 8 hairless mice during their growth from juvenile to adult (6–36 wk) and senescent life (up to 78 wk). Identical ear tissue regions were repetitively studied using intravital fluorescence microscopy for the analysis of age-related adaptations of the skin microcirculation. Exact locations for the measurement of intervascular distances were marked on the initially made video prints of the preselected tissue regions. Values are means ± SE. *P < 0.05, **P < 0.01 vs. 6 wk; ##P < 0.01 vs. 36 wk.

Fig. 4. Regression analysis between mean values of mice ear area and mean values of both the arteriolar (top) and the venular segment lengths (bottom). With intravital fluorescence microscopy, data were assessed by repetitive analysis of 8 hairless mice during their growth from late juvenile to adult and senescent life (6–78 wk). Values are means ± SE; r, regression coefficient.
Methodological considerations. The hairless mouse initially develops a full hair coat, but, at the age of 10–14 days, begins to lose hair on the face and legs. This continues until the whole head is bare. Shedding proceeds in a cephalocaudal direction until the animal is completely naked by 21 days of age (3). This pertinent hair loss guarantees perfect visualization of the microvasculature of the ear, which, per se, does not require any surgical preparation. Thus concerns regarding the effect of surgical procedures, such as laparotomy and exteriorization of the intestine (15, 17), on the normal growth of the animal as well as of the tissue of interest are negligible in the present study. Moreover, the fingerprint-like angioarchitecture of the skin does not require an artificial vessel marking, as must be necessarily performed using india ink dots for identification of intestinal segments of interest at a subsequent time point (15). Taken together, effects of surgery and tissue manipulation on age-related adaptations of the skin microvasculature can be considered minimal in this study, contrasting the methodological approach in previous studies analyzing the intestinal microvasculature in identical rats at 10 (late juvenile life) and 20 wk of age (early adult life) (17). Moreover, the present study extends previous studies (15, 17) in that repeated analysis of identical microvessels can be performed over time periods of several months up to 1.5 years (78 wk), thereby also covering the period from late adulthood to senescence. The fact that all measurements are confined to identical vessels throughout the whole experimental time period reduces potential variation of data due to intra-individual biological heterogeneity to a minimum and can be considered as a major advantage compared with longitudinal studies in animals of different ages (11, 13). Moreover, the present study encompasses the analysis of the whole microvascular network, including arterioles, capillaries, and venules, and does not focus only on the capillary arrangement (13) and the proximal or distal parts of the arteriolar tree (15, 17).

When measuring cutaneous capillarization, we used the functional capillary density as a measure of length of perfused capillaries per area of observation, contrasting parameters such as capillary path and segment lengths typically obtained in the skeletal muscle tissue (5, 13). The characteristic honeycomb-like arrangement of capillaries within the skin makes it inappropriate to define a capillary in accordance with the commonly used criteria as a vessel with its origin at the last division of the terminal arteriole and its terminus either at the point where it converges with another capillary to form a venule or where it joins a venule directly (4, 8). However, the herein presented

![Fig. 5. Regression analysis between mean values of mice ear area and mean values of both the arterioarteriolar (top) and venovenular distances (bottom). With intravital fluorescence microscopy, data were assessed by repetitive analysis of 8 hairless mice during their growth from late juvenile to adult and senescent life (6–78 wk). Values are means ± SE.](http://ajpheart.physiology.org/)
of 6 wk of age. Arterioles: diameter range 15.0–52.5 according to their initial diameter at the first observation time point of 6 wk of age (100%). Arterioles (20 measurement were marked on the initially made video prints of the preselected tissue regions. Vessels were grouped in accordance to their intravital fluorescence microscopy for the analysis of age-related adaptations of the skin microcirculation. Exact locations of beginning and end points for the measurement of vessel tortuosity were marked on the initially made video prints of the preselected tissue regions. Venules were the measurement of vessel tortuosity were marked on the initially made video prints of the preselected tissue regions. Venules were grouped in vessel classes of 15.0–52.5 μm, N = 15.

Values are means ± SE, **P < 0.01 vs. 6 wk.

parameter of functional capillary density estimates only those vessels actively serving for nutritive perfusion, rather than the maximum (anatomic) number of capillaries available for blood flow.

Age-related adaptations of the skin microvasculature. By comparison of the same vascular areas of mice ears at all ages studied, it is evident that during both maturation from late juvenile to adult life and aging to

Table 4. Diameter changes of arteriolar and venular microvessels of ears of 8 hairless mice during their growth from late juvenile to adult and senescent life (6–78 wk)

<table>
<thead>
<tr>
<th>Arteriolar diameter (μm)</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
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<tbody>
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<td>Arteriolar diameter (μm)</td>
<td>95.7</td>
<td>96.7</td>
<td>109.1</td>
<td>105.0</td>
<td>101.4</td>
<td>101.5</td>
<td>103.6</td>
<td>104.5</td>
<td>105.4</td>
<td>112.1</td>
<td>121.4</td>
<td>116.4</td>
<td></td>
</tr>
<tr>
<td>&lt;20 μm</td>
<td>100</td>
<td>4.1</td>
<td>5.3</td>
<td>7.5</td>
<td>6.3</td>
<td>6.8</td>
<td>3.8</td>
<td>7.4</td>
<td>7.8</td>
<td>7.6</td>
<td>8.1</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>&gt;20 μm</td>
<td>100</td>
<td>2.8</td>
<td>3.8</td>
<td>4.2</td>
<td>4.5</td>
<td>4.8*</td>
<td>5.5</td>
<td>5.2</td>
<td>8.1</td>
<td>6.3</td>
<td>14.6</td>
<td>7.7</td>
<td>11.8</td>
</tr>
<tr>
<td>Venular diameter (μm)</td>
<td>100</td>
<td>2.9</td>
<td>3.7</td>
<td>3.7</td>
<td>3.8</td>
<td>6.6</td>
<td>6.3</td>
<td>5.2</td>
<td>5.6</td>
<td>5.1</td>
<td>5.9</td>
<td>2.9</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Values are means ± SE in μm. Nos. in heading indicate week. Within the mice ears, identical tissue regions were repetitively studied using intravital fluorescence microscopy for the analysis of age-related adaptations of the skin microcirculation. Exact locations for the diameter measurement were marked on the initially made video prints of the preselected tissue regions. Vessels were grouped in accordance to their diameter at the first observation time point of 6 wk of age (100%). Arterioles (<20 μm), N = 18; arterioles (>20 μm), N = 17; venules (<40 μm), N = 18; venules (>40 μm), N = 20. *P < 0.05 vs. 6 wk.
The most striking effects of age-related adaptations of the skin microvasculature occurred during the late juvenile growth phase and early adult life. Compared with immature mice, the adult mice exhibited a greater mean ear area, mean arteriolar and venular segment length and volumetric blood flow, mean arterioarteriolar and venovenular distance, and mean venular tortuosity. Except for the venular tortuosity, all other parameters showed a slight decrease in mice from late adulthood to senescence. During aging, the increase of venular tortuosity seems to reflect a disproportional increase in length, in comparison with the ear growth, rather than a shrinkage/shortening of surrounding tissue. Tissue shrinkage, in turn, might be causative for the simultaneous reduction of ear size together with the dimensions of its supplying microvascular network during the time period from 36 wk of age to senescence. In addition to these “passive” adaptations of cutaneous arterioles and venules to the age-related changes of tissue mass, there is evidence for a need-oriented response of nutritive blood supply, i.e., recruitment and/or new formation of capillaries, during the late juvenile and early adult growth phase, thereby maintaining the functional capillary density and, thus, oxygen supply to tissue despite the increase of tissue area. This is in line with and extends previous studies by Unthank and co-workers (16, 17), who suggested capillary angiogenesis when comparing intercapillary distances for the longitudinal muscle layers of the rat intestine in 20- to 17- and 5-wk-old rats (16). The marked reduction of cutaneous functional capillary density with aging to senescence might also be interpreted in that age significantly modifies this microvascular parameter in relation to reduced oxygen demands. In addition to this morphological aspect, functional analysis of microvascular reactivity by studying the hyperemic response on short ischemia additionally revealed a restricted capacity of the microcirculation to produce adequate postischemic reactive hyperemia in old vs. young and early adult animals.

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