that extracts of retinas induced vasoproliferation. 4"6 of desmosomes among the ectodermal cells during the CAM development. The above results suggest that traction chorioallantoic membranes (CAMs) of the developing chick embryo were placed under tension with 10-0 nylon sutures in one phase of the study, CAMs from 6- to 8-day-old chick embryos were placed under traction and sampled after 1 day. As controls, some eggs had application of traction, and some eggs had a large stimulus.

In order to investigate the effect of traction of the vasculature on induction or acceleration of neovascularization, the chorioallantoic membranes (CAMs) of the developing chick embryo were placed under tension with 10-0 nylon sutures and subsequently examined histologically. In the initial phase of the study, CAMs from 6- to 8-day-old chick embryos were placed under traction and sampled serially. The cell proliferation and migration from the ectoderm to the mesoderm were observed from half a day to 3 days after the initiation of traction, and increase in the density of the mesodermal vessels was initially observed after 3 days. In the second study, CAMs from embryos at each age from 7 to 16 days were placed under traction and sampled after 1 day. As the CAMs developed, the ectodermal cell proliferation and migration decreased. This may be related with the increase of desmosomes among the ectodermal cells during the CAM development. The above results suggest that traction can induce the changes of the vasculature, and the effect of traction varies with the development of CAMs. Invest Ophthalmol Vis Sci 30:778-782, 1989.

Michaelson first proposed that a chemical factor in the retina controls the growth and development of retinal vasculature.1 Later, some authors suggested pathologic neovascularization was induced by chemical ischemia,2,3 and experimentally it was revealed that extracts of retinas induced vasoproliferation.4-6 Recently, it has been concluded from clinical observation that vitreous traction on the retinal vasculature serves as a mechanical stimulus for neovascularization in diabetic patients.7,8 However, the effect of traction on the vasculature has not yet been investigated experimentally. In this report, in order to investigate the effect of traction on the induction or acceleration of neovascularization, a tractional force was exerted on the chorioallantoic membranes (CAMs) of developing chick embryos and the vascular changes were observed histologically. CAMs have been used to study neovascularization, because the vasculature is easily affected by numerous stimuli.4-6,10

Materials and Methods. The CAMs of 6- to 17-day-old fertilized white Leghorn chicken eggs, incubated at 38°C, were used, in a manner that conformed to ARVO Resolution on the Use of Animals in Research. The fertilized eggs were set upright in an incubator at 38°C. On the 6th day after fertilization or later, a window was made in the eggshell overlying the air sac and the CAM was exposed. The CAM was placed under traction with 10-0 nylon suture in one direction. The shell window was sealed with cellophane tape, and the egg was replaced in the incubator at 38°C. The vascular changes of the CAMs were examined histologically. As controls, some eggs had windows but no other intervention, some eggs had a window and 10-0 nylon suture placed without the application of traction, and some eggs had a large

References
Fig. 1. Histological development of CAMs with eggshells opened and no other treatments. (Ec; ectoderm, M; mesoderm, En; endoderm). (a) Macroscopic view of 6-day-old CAM. Vascular network is noted. (b) Light micrograph of 6-day-old CAM. Capillaries are present in ectoderm and few blood vessels are noted in mesoderm (×125, toluidine blue staining). (c) Electron micrograph of ectoderm of 6-day-old CAM. There are only a few ectodermal cells around capillaries (C) and junctions among them are scarce. E: endothelial cell of capillary (×5700, bar = 1 μm). (d) Electron micrograph of ectoderm of 12-day-old CAM. There are more ectodermal cells and desmosomes (D) among them in comparison with 6-day-old CAM. Ectodermal cells have intermediate filaments (IF) in cytoplasm. Between the most superficial cells of ectoderm, junctional complex containing zonula occludens and zonula adherens is observed (JC) (×5700, bar = 1 μm). (e) Light micrograph of 17-day-old CAM. There are only a few blood vessels in mesoderm (×125, toluidine blue staining). (f) Electron micrograph of ectoderm of 17-day-old CAM. There are numerous ectodermal cells around capillaries and desmosomes (D) and microvilli (MV) among them are noted. Ectodermal cells have intermediate filaments (IF) in cytoplasm (×6600, bar = 1 μm).

hole in the CAM. The resulting vascular changes in these controls were studied in the same fashion as the CAMs which underwent traction. All specimens were fixed in a phosphate-buffered 2.5% glutaraldehyde solution (pH 7.4), postfixed in 2% osmium tetroxide solution, dehydrated in a graded series of ethanol, and embedded in Epok 812® (Oken Shoji, Tokyo, Japan). The sections were observed by light and transmission electron microscopy (JEM 1200EX).

The sampling timing and sequence differed be-
Fig. 2. CAM placed under tension with 10-0 nylon suture (Stage B and B'). (Ec; ectoderm, M; mesoderm, En; endoderm). (a) Light micrograph of Stage B CAM. Many cross-sections of small vessels (V) are noted. (x135, toluidine blue staining). (b) Electron micrograph of ectoderm of Stage B CAM. Numerous cells are noted around capillaries, but desmosomes among them were not observed. Many cytoplasmic organelles are noted in ectodermal cells. Between the most superficial cells of ectoderm junctional complex containing zonula occludens and zonula adherens (JC) is observed (x6000, bar = 1 \( \mu \)m). (c) Electron micrograph of ectoderm of Stage B' CAM. A few desmosomes (D) are noted among ectodermal cells. Many cytoplasmic organelles are noted in ectodermal cells (x8000, bar = 1 \( \mu \)m).

Results. Experiment I: The histological development of the CAMs in the control with the eggshells opened, but no other intervention is shown in Figure 1. The vascular network was noted in the CAMs macroscopically (Fig. 1a). In the 6-day-old CAM, capillaries were present in the ectoderm and few blood vessels were noted in the mesoderm by light microscopy (Fig. 1b). The electron micrograph of the ectoderm showed that there were only a few ectodermal cells around capillaries and junctions among them were scarce (Fig. 1c). Ectodermal cells and the desmosomes among them increased with the development of the chick embryos. By transmission electron microscopy, there were more cells around the capillaries and more desmosomes in the ectoderm of 12-day-old CAM in comparison with the 6-day-old CAM (Fig. 1c, d). Between the most superficial cells of the ectoderm of the 12-day-old CAM the junctional complex containing zonula occludens and zonula adherens was observed (Fig. 1d). The ectodermal cells of the 12-day-old CAM contained bundles of intermediate filaments in their cytoplasm. In the 17-day-old CAM only a few mesodermal vessels were observed by light microscopy (Fig. 1e). There were numerous ectodermal cells around capillaries and more numerous desmosomes among them in comparison with the 12-day-old CAM, as noted by transmission electron microscopy. The intermediate filaments in the cytoplasm of the ectodermal cells were noted and microvilli were observed among the ectodermal cells (Fig. 1f). The ectodermal cells of CAMs in the control did not contain so many organelles in the cytoplasm (Fig. 1c, d, f).

The CAM placed under traction with nylon suture exhibited three types of histological pattern and density of blood vessels. In this report, they are called Stages A, B, and C, and are related to the time course.
In Stage A, there was no significant difference from controls. In Stage B, cell proliferation and migration from ectoderm to mesoderm was observed. Many cross-sections of small vessels were noted in the mesoderm along the ectoderm in this stage (Fig. 2a). By transmission electron microscopy, numerous cells were noted around capillaries. Between the most superficial cells of the ectoderm the junctional complex containing zonula occludens and zonula adherens was observed. Desmosomes among the ectodermal cells around the capillaries were not observed. Many cytoplasmic organelles were noted in the ectodermal cells; however, intermediate filaments were not observed (Fig. 2b). In some Stage B CAMs, only slight cell proliferation and migration were found. By transmission electron microscopy, a few desmosomes among the ectodermal cells were noted and the ectodermal cells contained many cytoplasmic organelles. Microvilli among the ectodermal cells were not noted (Fig. 2c). This subgroup of Stage B is called stage B' in this report. In stage C, numerous large vessels in parallel with the direction of the traction were noted on the portion of the CAM placed under traction compared with the opposite side of the CAM (Fig. 3a). Numerous vessels in the mesoderm were observed in the light micrograph (Fig. 3b). Numerous desmosomes among the ectodermal cells were noted in the transmission electron micrograph. Plenty of intermediate filaments in the ectodermal cells and microvilli among the ectodermal cells were noted; however, cytoplasmic organelles in the ectodermal cells were few (Fig. 3c).

Time course of these changes is summarized in the Table 1. This Table shows the number of eggs in each stage for each duration of traction. A Stage B response was observed from half a day to 3 days after the beginning of traction, and a Stage C response was observed after 3 days or later. A Stage B' response was observed 3 days and 4 days after the beginning of traction.

Table 1. Stage of the response of the CAM blood vessels to traction which started on the 6th to 8th fertilized day

<table>
<thead>
<tr>
<th>Stage</th>
<th>Duration of traction (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
</tr>
<tr>
<td>B'</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers of eggs exhibiting indicated stage in each duration are shown.
traction. The vasculature of the CAMs sutured with 10-0 nylon suture but not placed under traction was not different from that of the CAMs from eggs with eggshell windows and no further treatment. The vasculature of the CAMs with large holes, a control designed to investigate the reaction of the CAM to the wound, was not different from the reaction of the CAMs with the eggshells opened and no other treatments.

Experiment II: When the 7- to 9-day-old CAMs were placed under traction, a Stage B response was observed after 1 day following the initiation of traction. When 10- to 12-day-old CAMs were placed under traction, stage B' changes were observed after 1 day. When 13-day-old or older CAMs were placed under traction, no vascular changes were noted after 1 day.

Discussion. In the CAMs placed under traction with 10-0 nylon suture, ectodermal cells seemed to be activated, with an increase of cytoplasmic organelles and decrease of cytoplasmic intermediate filaments and intercellular junctions compared to controls in early stage, and later the density of mesodermal blood vessels was observed to increase. These results suggest that traction exerted on the CAMs induced the cell proliferation and migration observed in the Stage B response and the increase in the mesodermal vessel density observed in the Stage C response. As shown in Table 1, a Stage B' response was observed 3 to 4 days after the initiation of traction, at a time between that expected for a Stage B and Stage C response. This Stage B' may be a transient stage between Stage B and Stage C in Experiment I. These results cannot be immediately applied to the retinal vasculature in diabetic patients, but suggest that the traction on the vessels may provide a stimulus for vascular growth. The results of Experiment II suggest that the response of the CAM vasculature to traction differs according to the age of the CAM when the traction is applied. As the CAMs became older, the ectodermal cell response decreased. As shown in Figure 1, illustrating the control with only the eggshell window, desmosomes among the ectodermal cells around capillaries appeared to increase with the development of chick embryos. Junctional complexes containing zona occludens and zona adherens were observed among the most superficial cells of the ectoderm both with and without traction. Desmosomes are known to be sites of cell-to-cell adhesion, and this increase of desmosomes along the histological development may be related to the results of Experiment II. Tractional force alone cannot break the well developed intercellular junctions among the ectodermal cells. Retinal neovascularization is rarely observed even by histological examination in patients with proliferative vitreoretinopathy or macular pucker, which suggests that traction on the retina alone cannot induce neovascularization. This clinicopathological observation and the results of Experiment II suggest that traction on the vasculature is not an "angiogenesis factor," but a factor which may accelerate the neovascularization from the vulnerable vessels, in other words, a factor previously activated by other chemical factors.

Key words: chorioallantoic membranes, traction, changes of vasculature, histological examination

From the *Department of Ophthalmology, University of Tokyo School of Medicine, and the †Department of Ophthalmology, Tokyo Women's Medical College Diabetes Mellitus Center, Tokyo, Japan. Presented in part at the annual meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Florida, May 1-6, 1988. Submitted for publication: June 1, 1988; accepted October 24, 1988. Reprint requests: Hidetoshi Yamashita, MD, Department of Ophthalmology, University of Tokyo School of Medicine, Hongo 7-3-1, Bunkyo-ku, Tokyo, 113, Japan.

References