Apoptosis in Surgically Excised Choroidal Neovascular Membranes in Age-Related Macular Degeneration

David R. Hinton, MD; Shikun He, MD; Pedro F. Lopez, MD

Background: Choroidal neovascular membranes (CNVMs) in age-related macular degeneration show progressive histologic changes from active, cellular, highly vascularized membranes to inactive paucicellular scars. The purpose of this study was to determine whether apoptosis was involved in the evolution of these changes, what cell types are involved, and whether there was an association with the Fas antigen (Fas or CD95) and Fas ligand (FasL).

Methods: Serial frozen sections from 10 surgically excised CNVMs were stained by the TUNEL (terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine 5-triphosphate nick-end labeling) method for detection of DNA strand breaks and by propidium iodide staining for morphologic detection of apoptosis. Immunoperoxidase staining was used for detection of Fas, FasL, and cell-type specific antigens.

Results: Highly vascularized membranes contained cells with TUNEL-positive nuclei, particularly in the regions of neovascularization, while fibrotic membranes showed few, if any, TUNEL-positive cells. Many of the TUNEL-positive cells were stromal retinal pigment epithelial cells, although smaller numbers were identified as endothelial cells and macrophages. Confocal microscopy of propidium iodide–stained sections confirmed the presence of apoptotic nuclei. The extent of Fas antigen expression correlated with extent of apoptosis. FasL expression was found in all specimens but was most intense in the highly vascularized membranes.

Conclusions: Highly vascularized CNVMs related to age-related macular degeneration show apoptosis in stromal retinal pigment epithelial cells, endothelial cells, and occasional macrophages. Apoptosis is associated with prominent Fas and FasL expression.


HOROIDAL neovascular membranes (CNVMs) are initiated by the migration of choroidal endothelial cells through the Bruch membrane into the subretinal pigment epithelial space. Angiogenesis continues with the recruitment of other cell types and extension into the subretinal space. The cellular constituents of these cellular CNVMs include retinal pigment epithelial (RPE) cells, which migrate from the RPE monolayer, where they often become positive for smooth-muscle actin (SMA), and express basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Also present are smaller numbers of macrophages and keratin-negative myofibroblasts. The membrane may become adherent to the neural retina by means of a glial scar; however, the astrocytes do not infiltrate into the membrane. The CNVM at this stage has many of the characteristics of granulation tissue, suggesting that it may demonstrate features of a stereotyped wound-healing response. Further evolution of the lesion includes decreasing cellularity and the production of collagen, resulting in a fibrous scar.

The mechanism of the evolution of this vascular lesion into a scar has not been elucidated; however, it is likely that there will be parallels with mechanisms identified in other wound-healing situations. Recently, it has been suggested that apoptosis mediates the decrease in cellularity found during the transition between granulation tissue and fibrous scar after experimental skin wounding. Apoptosis is a unique form of cell death that can be differentiated from necrosis by morphologic and biochemical means; in the eye, apoptosis has been identified in normal retinal development, retinal detachment, several inherited retinal degenerations, and primary open-angle glaucoma. Retinal pigment epithelial apoptosis has been seen after light damage in vivo and after experimental ischemia in vitro. Apoptosis has also been previously used in the retinal literature as a...
MATERIALS AND METHODS

TISSUE PREPARATION

Surgical excision of ARMD-related, subfoveal CNVMs was performed in 10 eyes from 10 patients. All procedures were performed by one of us (P.F.L.). Two of the patients had undergone previous foveal laser photocoagulation. The tenets of the Declaration of Helsinki, Finland, were followed—inform consent was obtained, and institutional human experimentation committee approval was granted for this study. Seven of the 10 membranes had been evaluated previously in a study examining the cellular components and the presence of VEGF expression in ARMD-related CNVMs. The indications for this surgery and the techniques employed have been reported previously. The basic characteristics of the excised membranes, including their appearance on a fluorescin angiogram, are described in the Table. The Macular Photocoagulation Study classification of each choroidal neovascular lesion as either a classic or occult CNVM (<25% white fibrous tissue) or as a CNVM/scar (>25% white fibrous tissue) was ascertained. Both of the occult lesions showed fibrovascular pigment epithelial detachment.

Each of the fresh, surgically excised CNVMs was placed in isotonic saline solution at 4°C, then snap-frozen in optimum cutting temperature compound (Ames/Miles Inc, Elkhart, Ind) within 1 hour. Chorioretinal specimens from fresh, age-matched, postmortem donor eyes without evidence of chorioretinal disease were obtained within 12 hours of death and were processed similarly to the normal human control specimens. Each specimen was serially sectioned on a cryostat into 6-µm frozen sections on glass slides coated with poly-L-lysine (Sigma, St Louis Mo) for immunohistochemical and TUNEL (terminal deoxynucleotidyl transferase–mediated biotin-deoxyuridine 5-triphosphate nick-end labeling) staining. The sections were fixed in 1% paraformaldehyde for 5 minutes at room temperature and stored at −80°C. Hematoxylin-eosin–stained adjacent sections were evaluated to determine the histologic diagnosis and to ensure tissue quality and orientation.

PROPRIODIUM IODIDE STAINING

Thawed tissue sections were rinsed in phosphate-buffered saline (pH 7.4) after fixation in 4% paraformaldehyde for 10 minutes. The sections were then stained with a mixture of the intercalating DNA-binding dye, propidium iodide (1 µg/mL, Sigma), and RNase (5 mg/mL, Sigma) for 10 minutes at 37°C. Sections were examined using a confocal laser scanning microscope with a rhodamine filter.

IMMUNOPEROXIDASE STAINING

Thawed tissue sections were air-dried, rehydrated with phosphate-buffered saline (pH 7.4), and incubated with blocking serum for 15 minutes. Sections were incubated for 30 to 60 minutes with primary antibody then washed for 15 minutes with phosphate-buffered saline. Immunoperoxidase detection was performed using the ABC Elite kit (Vector, Burlingame, Calif) with aminoethylcarbazole as the red chromogen. Slides were then rinsed with tap water, counterstained with hematoxylin, and mounted in aqueous mounting medium.

TERM TO DESCRIBE THE EREXION OF RPE CYTOPLASM INTO THE BRUCH MEMBRANE IN THE FORMATION OF DRUSEN, BUT THIS USAGE HAS LARGELY BEEN REPLACED BY THE TERM “BUDDING,”. Apoptosis does not result in an inflammatory response, because dead cells are phagocytosed by adjacent cells. Such a process is advantageous in the outer retina, where inflammation could result in further damage to the sensitive neural structures. Apoptosis is usually an active process and is distinctively associated with DNA fragmentation by endonucleases, a process that allows for in situ identification of apoptotic nuclei by labeling the newly formed 3’-hydroxy ends.

Table

<table>
<thead>
<tr>
<th>Patient No./Age, y/ Race/Sex</th>
<th>MPS Classification</th>
<th>Location†</th>
<th>Neovascularization‡</th>
<th>Fibrosis§</th>
<th>Apoptosis¶</th>
<th>TUNEL</th>
<th>PI</th>
<th>Fas</th>
<th>FasL</th>
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</thead>
<tbody>
<tr>
<td>1/63/W/F CNV/scar</td>
<td>Subretinal and sub-RPE</td>
<td>+++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2/70/M/F CNV/scar</td>
<td>Subretinal</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/71/W/F CNV/scar</td>
<td>Subretinal</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/72/W/F CNV/scar</td>
<td>Subretinal</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
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<tr>
<td>5/84/W/F Recurrent classic and occult CNV</td>
<td>Indeterminate</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/71/W/F CNV/scar</td>
<td>Subretinal</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
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<tr>
<td>7/71/W/F Recurrent CNV/scar</td>
<td>Indeterminate</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
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</tr>
<tr>
<td>8/77/W/F CNV/scar</td>
<td>Subretinal</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/65/W/F Occult CNV</td>
<td>Subretinal</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>+++</td>
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<tr>
<td>10/67/W/F Classic CNV</td>
<td>Subretinal and sub-RPE</td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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</tr>
</tbody>
</table>

* MPS indicates Macular Photocoagulation Study; CNV, choroidal neovascularization. Occult CNV lesions show fibrovascular pigment epithelial detachment.
† RPE indicates retinal pigment epithelium.
‡ By analysis of hematoxylin-eosin stain and immunoperoxidase stain for human von Willebrand factor. Plus sign indicates infrequent individual vessel; ++, moderate number of vessels; and ++++, frequent vessels.
§ Percent area on histologic section. Plus sign indicates 10%-40%; ++, 40%-70%; and ++++, 70%-100%.
¶ TUNEL indicates terminal deoxynucleotidyl transferase–mediated biotin-deoxyuridine 5-triphosphate nick-end labeling; PI, propidium iodide.

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with glycerin-gelatin medium. Staining for each antigen was performed at the same time to ensure consistency between specimens.

Monoclonal mouse antibodies against cytokeratin 18 (CK18) (1:400 dilution, Sigma), CD68 (1:100 dilution, Dako, Carpinteria, Calif), and Fas (Medical and Biological Laboratories CD Ltd, Watertown, Mass) and polyclonal rabbit antibodies against human von Willebrand factor (1:100 dilution, Dako) and FasL (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) were used.

Biotinylated secondary antibodies (1:400 dilution) were obtained from Vector. FasL staining was performed as described by Griffith et al. Antibody concentrations were individually determined on appropriate positive control tissues. Negative controls included omission of primary antibody and use of an irrelevant primary antibody of the same isotype. Cytocentrifuge preparations of positive (tumor necrosis factor α-stimulated RPE cells) and negative (fibroblasts or unstimulated fetal human RPE) control cells were stained in parallel with the CNVM slides.

TUNEL STAINING

Thawed frozen tissue sections were hydrated with phosphate-buffered saline for 5 minutes. The TUNEL staining was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, Md) according to the supplied protocol for cryosections. In this method, cut DNA is localized in situ by end-labeling, using terminal deoxynucleotidyl transferase (TdT) and a digoxigenin–deoxyuridine 3′-triphosphate substrate. The bound digoxigenin is detected immunohistochemically using antidigoxigenin-peroxidase antibodies. Peroxidase was visualized using aminoethylcarbazole as the chromogen (AEC Kit, Zymed Laboratories Inc, South San Francisco, Calif). Negative controls included substitution of water for TdT.

DOUBLE IMMUNOPEROXIDASE AND TUNEL STAINING

Thawed frozen tissue sections were fixed for 10 minutes with 10% neutral buffered formalin (Polyscience Inc, War- rington, Pa). They were then washed 3 times with Tris hydrochloride buffer (pH 7.4) for 5 minutes and blocked with 5% normal goat serum for 15 minutes. Primary antibody (CD68, CK18, or von Willebrand factor, at dilutions listed earlier) was then added to cover the tissue and incubated for 1 hour at room temperature.

The sections were washed 3 times, with Tris hydrochloride buffer and secondary biotinylated antimouse or antirabbit antibodies (Vector) added for 30 minutes. Sections were washed 3 times with Tris hydrochloride buffer for 5 minutes; streptavidin-phosphatase (Kirkegaard and Perry Laboratories Inc, Gaithersburg, Md) was then added for 25 minutes. Another 3 washes with Tris hydrochloride buffer were followed by a 5- to 10-minute incubation with 1 drop (≈20 μL) of HistoMark Blue, prepared from the HistoMark Blue Kit (Kirkegaard and Perry Laboratories Inc). The slides were washed with phosphate-buffered saline for 5 minutes, then fixed again with 10% neutral-buffered formalin for 10 minutes. The sections were washed with phosphate-buffered saline and detection of apoptosis was performed using the ApopTag in situ detection kit as described earlier.

Apoptosis is a complex multistep process for which many inducers and inhibitors have been identified. Of particular interest in scar formation is Fas, also known as CD95. Fas is a 36-kd cell-surface protein in the nerve growth factor/tumor necrosis factor receptor superfamily. When signaled by the Fas ligand (FasL), Fas mediates apoptosis in a variety of human cells and tumors by initiating a death signal that probably involves proteases similar to intracellular interleukin 1–converting enzyme. Fas is not constitutively expressed in the normal retina. Previous studies using pancytokeratin antibody showed that many of the stromal cells in both cellular and fibrotic membranes were cytokeratin positive, indicating their origin from RPE cells. In this study, we used CK18, which preferentially labels migrating RPE cells; the resting RPE monolayer is not reactive. The number of CK18-positive cells found within the stroma was similar to the number of cells labeled with pancytokeratin (data not shown).

The TUNEL stain labeled variable numbers of nuclei in 9 of 10 specimens (Table). In the cellular membranes, moderate to large numbers of individually positive cells were identified within the stroma, primarily in a perivascular distribution (Figure 1, A–C). In the fibrotic membranes only very rare TUNEL-positive nuclei were identified (Figure 1, D). There were no TUNEL-positive cells within either the intact RPE monolayers or the adherent retinal glial plaques in any specimen. Double-staining revealed that the majority of the TUNEL-positive cells were also positive for CK18 (Figure 1, E). Occasional von Willebrand factor–positive endothelial cells were also TUNEL positive (Figure 1, F), as were rare CD68+ macrophages (data not shown).

The surgically excised tissues varied in appearance from moderately cellular membranes with prominent neovascularization to paucicellular membranes with no demonstrable vascular channels (Table). A partially intact RPE monolayer was found in 8 of 10 specimens. Using the monolayer for orientation, 5 of the specimens were found to be subretinal, while 3 were both sub-RPE and subretinal. Previous studies using pancytokeratin antibody showed that many of the stromal cells in both cellular and fibrotic membranes were cytokeratin positive, indicating their origin from RPE cells. In this study, we used CK18, which preferentially labels migrating RPE cells; the resting RPE monolayer is not reactive. The number of CK18-positive cells found within the stroma was similar to the number of cells labeled with pancytokeratin (data not shown).

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Confocal microscopy of adjacent sections stained with propidium iodide revealed the presence of nuclei with morphologic features of apoptosis in 7 of 10 specimens (Table). Apoptosis was morphologically confirmed by the identification of shrunken nuclei with clumped chromatin forming crescents along the nuclear envelope (Figure 2, A), or by the presence of apoptotic bodies (Figure 2, B). The number of apoptotic cells correlated with the number of TUNEL-positive cells except that the number of apoptotic cells was generally lower (Table). Two of the specimens showing only small numbers of TUNEL-positive nuclei did not contain cells with apoptotic morphology using these criteria (Figure 2, C).

Fas antigen expression in the CNVMs ranged from strong (Figure 3, A and B) to absent (Figure 3, C). The level of Fas staining showed a highly significant correlation with the level of apoptosis identified ($r=0.98; P<.001$, Spearman correlation coefficient). Fas antigen was identified primarily on large epithelioid cells (Figure 3, B) with the appearance of transdifferentiated RPE, many of which were shown on adjacent and double-stained sections to be CK18 positive. Double staining showed that many of the cells undergoing apoptosis were Fas positive (data not shown). In the CNVMs, the remaining RPE monolayer was predominantly Fas negative but occasionally showed focal positivity (Figure 3, B). Control eyes were immunohistochemically negative for Fas expression in the RPE monolayer (Figure 3, D).

FasL expression was present in the normal eye throughout the neural retina, consistent with previous reports in rodents, but was only weakly expressed in the RPE monolayer (Figure 4, A). All of the CNVMs showed FasL expression in almost all of the stromal cells in the membrane and in residual RPE monolayer (Figures A, B).
ure 4, B and C). Perivascular stromal cells in active membranes, previously identified as RPE, were intensely stained. Almost all of the fibroblastic-appearing cells in fibrotic nonvascular membranes were FasL positive; however, the staining was only weak and focal. No significant correlation was present between FasL expression and the extent of apoptosis.

**COMMENT**

The evolution of a fibrous scar in ARMD-related CNVMs is critical to the pathogenesis of the disorder and may be important in the progressive degeneration of the overlying neural retina. We show here that apoptosis is commonly seen in the cellular, neovascular regions of ARMD-related CNVMs, and thus it is likely that the decrease in cellularity seen in the evolution of these lesions is mediated, at least in part, by this process. Further support for this notion is provided by the finding that the surgically excised CNVMs contain only small numbers of inflammatory cells, as is characteristic of tissues showing apoptotic cell death. Apoptosis is an appropriate method of cell reduction in the subretinal space, a location where an intense inflammatory infiltration could lead to even more severe retinal injury. Our findings provide more evidence supporting the view that ARMD-related CNVMs...
have many of the features of a stereotyped wound-healing response, because granulation tissue shows reduction of cellularity in a similar manner.8

Apoptosis was implied and most easily quantitated by the presence of TUNEL positivity but was confirmed by the finding of cells with typical apoptotic morphology, using the DNA intercalating dye propidium iodide. While further documentation of apoptosis by means of a DNA laddering pattern may have been ideal, this was not practical considering the very small sample size available from these surgical specimens.

Double-staining experiments revealed that the predominant cell type undergoing apoptosis was cytokeratin positive and thus, in this site, represents RPE, a cell type that is a major constituent of the ARM-related CNVM stroma. These cells have been shown to be positive for SMA and have been previously described as transdifferentiated RPE or myofibroblasts with the potential for contractile activity.3,5 It is intriguing that in the study of apoptosis in granulation tissue and atherosclerosis, apoptosis has been predominantly localized to SMA-positive myofibroblasts and smooth muscle cells in these tissues. The specific reason for the apparent susceptibility of these SMA-positive cell types to apoptosis is not clear.

In ARM-related CNVMs, the transdifferentiated RPE cell is also critical for its production of angiogenic factors such as VEGF. Selective apoptosis of these VEGF-producing cells may ultimately mediate the devascularization of the CNVM; however, this effect may be modulated or delayed by the release of other angiogenic factors. Similar CNVM stromal cells have also been shown to be the source of bFGF. The difference between bFGF and VEGF is that bFGF is not secreted because there is no signal transduction sequence; the death of bFGF-producing cells by apoptosis could therefore result in the release of this angiogenic factor and at least temporarily provide a continued stimulus for angiogenesis. It has also been shown that bFGF delays photoreceptor cell degeneration, presumably due to apoptosis, in rats with inherited retinal dystrophy, and also reduces the photoreceptor degeneration produced by constant light exposure. In a similar manner, the released bFGF could potentially delay apoptosis in the photoreceptors of the overlying neural retina detached from the RPE layer by the subretinal CNVM.

We have shown that apoptosis also occurs to a lesser extent in von Willebrand factor–positive endothelial cells, thus allowing for devascularization of the CNVM. Apoptosis of endothelial cells has been shown in certain situations, such as experimental retinopathy of prematurity, to be temporally associated with a decrease of VEGF activity. Vascular endothelial growth factor supports the growth and maturation of endothelial cells and its decrease signals the previously supported cells to undergo apoptosis. In a similar way, developing dorsal root ganglion sensory neurons undergo apoptosis in response to withdrawal of nerve growth factor. It is not clear whether the apoptosis of endothelial cells in ARM-related CNVMs is due to loss of growth factors when RPE cells die or to the same signal that initiates stromal RPE apoptosis. It has also been shown recently that RPE cells express VEGF receptors and may be subject to regulation by this growth factor. Apoptotic loss of transdifferentiated RPE cells may therefore potentially stimulate further loss of RPE cells and endothelial cells by this mechanism. While the number of ARM-related CNVMs in this study is insufficient to address this question, it is intriguing that the CNVM with the most numerous apoptotic cells was previously shown to have very low VEGF expression.

Apoptosis has been shown to be an important mechanism of cell death in retinal development and in several retinal degenerations. In these models there is predominant loss of photoreceptors; the RPE cell population, however, did not show any evidence of apoptosis. In experimental light damage, the RPE cells undergo apoptosis and, in contrast to the photoreceptors, they show coincident development of TUNEL positivity and apoptotic morphology, again providing support for our use of these complementary methods. Fas and FasL interactions are a likely mechanism for signaling apoptosis in RPE cells contained within the CNVM stroma. Fas was highly expressed in the CNVMs where apoptosis was present and was localized to cells with the appearance of transdifferentiated RPE. Residual RPE monolayers within the surgically excised specimens were predominantly Fas negative and the RPE monolayer of control eyes was Fas negative. It has been shown that RPE cells in culture show weak expression of Fas that is significantly increased with cytokine stimulation by tumor necrosis factor or interferon γ. The lack of expression in retinal sections may be due to a lower sensitivity of immunohistochemical analysis when compared with flow cytometry, or may be due to the fact that the act of culturing RPE cells may be sufficient to minimally stimulate some cell surface molecules. The up-regulation of RPE Fas expression by inflammatory cytokines in vitro suggests that they, or perhaps other stimuli, are at least transiently present in ARM-related CNVMs. Signaling through Fas requires the presence of FasL, which is characteristically present on activated T cells; however, T cells are very rare in these membranes (data not shown). Recently it has been shown that in the rodent eye, FasL is expressed in the cornea, iris, ciliary body, and throughout the retina and that this expression may be an important mechanism for maintenance of immune privilege. Our demonstration of FasL expression in the human retina was consistent with the findings in rodents. FasL expression was found in all CNVMs; although the intensity of staining was least in those membranes that were fibrotic, there was no statistical correlation in vascular membranes between the amount of FasL expression and the degree of neovascularization. The detailed mechanism of Fas and FasL interactions in the retina has yet to be explored, and may be dependent on several factors, including Fas receptor density. A potential role for a soluble form of FasL must also be considered. Our findings suggest that in the retina the dramatic up-regulation of Fas, as we find in these ARM-related CNVMs in the presence of FasL, may result in a potent signal for apoptosis in those cells. The possibility that Fas may also have other relevant functions should be considered; in an experimental inflammatory angiogenesis model, stimulation of Fas triggered neoangiogenesis and infiltration of inflammatory cells independent of apoptosis.

The demonstration that apoptosis is commonly found in the active stages of ARM-related CNVMs provides a
link to other wound-healing responses. The retina is uniquely positioned to allow for rapid induction of apoptosis through Fas and FasL interactions because of the constitutive expression of FasL in this tissue. This mechanism may allow for noninflammatory resolution of the neovascular tissue into a fibrovascular scar. This form of healing is not without consequence, because the fibrovascular scar tissue may prevent diffusion of nutrients to the outer retina and result in progressive retinal damage. Further understanding of the mechanisms involved in subretinal scar formation may potentially allow for pharmacologic manipulation and resolution of ARM-related CNVMs.

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