Submicroscopic Distribution of Ruthenium Red in Human Glioblastoma Multiforme*

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Electron microscope studies have suggested the presence of a poly-anionic intercellular substance in mammalian central nervous tissue.\(^3\) The composition of this material, which appears to occupy the extracellular space of the brain, is not known, but chemical studies indicate the presence of an acid mucopolysaccharide.\(^20\) Its intercellular distribution has been demonstrated by visualization in the electron microscope of electron-opaque metal complexes (for example, of phosphotungstic acid\(^21\) and ruthenium red\(^4\)), but the relation of these complexes to plasma membranes is obscured in central nervous tissue by the sparsity of intercellular space. The dimensions of the extracellular space in brain are not known with certainty, and the stainable material localized within the intercellular spaces may be either a plasma membrane-component (cell coat) or a true intercellular substance.

The electron microscope study of glioblastoma multiforme reported here concerns the relationship of neoplastic cells to their immediate extracellular environment. Although it is recognized that there is no a priori relationship between the intercellular substance of the tumor and that described in normal brain, this study represents an attempt to differentiate plasma membrane and intercellular components in the central nervous system.

The term "glioblastoma multiforme" connotes a heterogeneous group of morphologically complex tumors, which because of similarities of histology and clinical course may be defined as a clinical entity. The morphological complexity of glioblastoma multi-

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Materials and Methods

Specimens of nine suspected gliomas were obtained during operation at the Neurosurgical Clinic, Sahlgrenska Hospital, Gothenburg, Sweden. After surgical exposure of the neoplasm, a specimen was taken by sharp dissection from an area thought not to be necrotic. Electrocautery was avoided and special care was exercised in the use of clamps and hemostats. Within 2 to 3 minutes after excision, the specimen was cut into strips about 3 to 5 mm by 0.3 to 0.5 mm, which were immersed in one of four fixatives. In the appropriate fixative the specimens were cut into cubes about 0.3 to 0.5 mm on a side. In each case, other specimens were fixed in formalin and processed for routine neuropathological diagnosis.

Specimens of each tumor were processed according to the following procedures.

Glutaraldehyde. Initial fixation was accomplished by immersion in 3% glutaraldehyde in sodium cacodylate buffer adjusted to pH 7.4 at 0°C for 1 hour. Following two 5-min washes in the cacodylate buffer, the specimens were additionally fixed in 1% OsO\(_4\), prepared according to Caulfield,\(^7\) for 1 hour at 0°C.

Osmium Tetroxide. Specimens were fixed by immersion in 1% OsO\(_4\), prepared according to Caulfield\(^7\) at 0°C for 1 hour.

Ruthenium Red. Specimens were fixed in a solution containing equal parts of 3% glutaraldehyde in cacodylate buffer (pH 7.3), sodium cacodylate buffer adjusted to pH 7.3,
and 0.25% aqueous ruthenium red, for 90 min at O°C. Following two 5-min washes in the buffer, the specimens were transferred to a solution containing equal parts of 0.25% aqueous ruthenium red, cacodylate buffer adjusted to pH 7.3, and 5% aqueous OsO₄ for 3 hours at room temperature in the dark. This procedure was described initially by Luft and has been used by Bondareff for the demonstration of extracellular substance in rat brain.

_Lanthanum Nitrate_. Specimens were fixed by immersion in a solution containing 1% \( \text{La(NO}_3\text{)}_3 \cdot 6\text{H}_2\text{O} \) and 1% \( \text{KMnO}_4 \) as described by Lesseps for 1 hour at O°C.

In each case, following fixation, the tissues were dehydrated in a graded series of ethyl alcohol, immersed in propylene oxide and embedded in Araldite 502.

Of the nine cases biopsied, a pathological diagnosis of glioblastoma multiforme was returned for seven. Although viable tumor tissue was indicated by gross inspection, it was not always possible to avoid sampling necrotic or hemorrhagic areas. Because such areas were often of microscopic dimensions, tissues were sectioned first at 0.5 to 0.1 \( \mu \), stained with methylene blue-Azure II, and examined light microscopically. Selected areas of these were cut on a Sorvall Ultratome or an LKB Ultratome and examined in a Siemens Elmiskop IA electron microscope. Specimens fixed with solutions containing ruthenium red or lanthanum nitrate were examined unstained. Specimens fixed in glutaraldehyde or osmium tetroxide were stained prior to viewing with lead citrate and uranyl acetate. Images were recorded on DuPont Cronar film and developed in D-19.

**Results**

_Glutaraldehyde-OsO₄ and OsO₄ Fixation_. The cytoplasm of well-preserved cells were dense with closely-packed organelles (Fig. 1 _top_). Many ribosomes occurred free in the cytoplasm, usually arranged in rosettes of 4 to 8 ribosomes, and the nucleus was often large and irregular in outline. These cells, and especially their processes, contained many closely-packed fibrils, about 70 \( \AA \) in diameter. There were many cytoplasmic dense bodies (Fig. 1 _top_), but the specific nature of these pleomorphic structures was not determined. They often appeared to be continuous with the membranes of the endoplasmic reticulum, and contained complex membranous configurations often resembling myelin figures.

The cells of glioblastoma multiforme characteristically possessed many tortuous processes, which apposed one another at varying distances (Fig. 1 _top_). The extracellular space was, therefore, of variable dimensions, being several microns wide in some places and only 100 to 200 \( \AA \) wide in others (Fig. 1 _top_). Apposing plasma membranes did not appear to fuse, and membrane configurations resembling tight junctions, close junctions, or desmosomes were not found.

Plasma membranes were typically trilaminar (Fig. 2 _bottom_), and the outer surface of the external leaflet was either smooth or associated with a variable amount of indistinct, fibrillar material, which was not uniformly distributed (Figs. 1 _bottom_ and 2 _top_ and _bottom_). The appearance of plasma membranes did not differ noticeably in specimens fixed with glutaraldehyde-OsO₄ or in those fixed with OsO₄ only.

The immediate external environment of the parenchymal tumor cells contained many, thin, non-collagenous fibrils (about 20 \( \AA \) in width), which appeared as single strands or aggregates of variable dimension (Fig. 1 _bottom_), and thicker fibrils (100–200 \( \AA \) in width), in which two limiting surfaces were apparent in longitudinal sections (Fig. 2 _top_). These different fibrils were intermingled and variously associated with one another. They appeared to be similar to the microfibrils described in normal and pathological connective tissues. Microfibrils in glioblastoma multiforme showed no particular distribution except at the cell surface where they often formed bundles organized with their long axes lying parallel to the plane of section (Fig. 1 _bottom_). They were, in such cases, always distinct from the plasma membrane and did not appear to be an integral part of it.

_Ruthenium Red_. A dense, granular reaction product was found in a zone about 1 \( \mu \) thick immediately subjacent to the surface of the specimen, in which the cells were often
Fig. 1. Top: Electron micrograph of human glioblastoma multiforme showing the spatial relationship of parenchymal tumor cells and their processes to the extracellular space. Glutaraldehyde-OsO₄ fixation. Uranyl acetate and lead citrate stain, ×16,000. Bottom: Surface of parenchymal tumor cell and the extracellular space. Extracellular fibrillar material, composed in part of fine microfibrils (arrows) which tend to accumulate at the cell surface. cdب = cytoplasmic dense bodies, M = mitochondria, N = nucleus, E = extracellular space, pv = micropinocytotic vesicles. Glutaraldehyde-OsO₄ fixation. Uranyl acetate and lead citrate stain, ×80,000.
Fig. 2. Top: Interference between tumor cell (cyt) and extracellular space. Note the sparsity of membrane "fuzz" associated with the outer leaflet of plasma membrane. Glutaraldehyde-OsO₄ fixation. Uranyl acetate and lead citrate stain, ×160,000. Bottom: Interface between tumor cell (cyt) and extracellular space. The relative sparsity of "fuzz" associated with the outer leaflet of plasma membrane (arrows) is demonstrated. cyt = cytoplasm of tumor cell, mf = microfibrils in extracellular space seen in longitudinal section. Glutaraldehyde-OsO₄ fixation. Uranyl acetate and lead citrate stain, ×200,000.

damaged mechanically. In this zone, plasma membranes were often not intact, and the dense ruthenium red reaction product was found intracellularly as well as extracellularly. Beneath this subsurface zone there was a layer, about 5 μ thick, in which plasma membranes were intact, and in which the dense ruthenium red reaction product was exclusively extracellular (Fig. 3 top). Below this, there was little penetration of ruthenium red, and no reaction product was found. In cases where cell processes were closely apposed, and the intercellular space only 150 to 200 A in width, the ruthenium red reaction product completely filled the intercellular space (Fig. 3 top). Where the in-
tercellular space was wider, the distribution of the ruthenium red reaction product was more variable but appeared to coincide with that of the microfibrils (Fig. 3 bottom). In all cases, the density of the intercellular substance was greatly enhanced by the reaction product, and the limits of the plasma membrane, which was clearly demarcated in these preparations, were quite clear and distinct from the intercellular substance.

Permanganate-Lanthanum Nitrate. A densely staining intercellular substance was demonstrated (Fig. 4). In these preparations there was no preferential staining of the external leaflet of plasma membrane, and no layer of membrane-associated lanthanum-staining material was found. 14

Discussion
Glioblastoma multiforme is a malignant neoplasm with a relatively large amount of intercellular material, not uniformly distributed. Staining reactions suggest that this intercellular material may contain mucopolysaccharide or glycoprotein, 1 but reactive materials could not be localized precisely. To what extent intracellular organelles might contribute to the apparently extracellular distribution of reactive substances visualized with the light microscope is not known with

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certainty because of the limited resolving power of light microscope methods. Lyso-
somes, present in the attenuated processes of glioblastoma cells (Fig. 1 top), possess staining
properties similar to those of extracellular components. In thick sections viewed
with the light microscope, in which cell boundaries are not defined accurately, these
intracellular organelles may contribute to staining reactions which artifactually appear
to be extracellular.

The electron-opaque particulates resulting from the ruthenium red reaction were selec-
tively distributed extracellularly in glioblastoma multiforme. The particulate reaction
product was found in those intercellular spaces in which microfibrils were demonstrat-
ed. The reaction appeared to involve either the microfibrils or the intercellular matrix with which these microfibrils occur. In either case, the reaction involved extracellular structures and presumably indicates the presence of mucopolysaccharide or glyco-
protein. Ruthenium red has been shown to react selectively with the extracellular matrix of cartilage, a tissue site known to contain abundant acid mucopolysaccharide, and with the mucopolysaccharide (heparin) of mast cell granules.

This study shows also that the ruthenium red-staining material of glioblastoma multi-
forme is not associated only with the "glycocalyx" of plasma membranes or the cell coat which appears to be demonstrated in normal rat brain by the periodic acid-silver methenamine reaction. Plasma membranes of the tumor cells were typically trilaminar and appeared essentially symmetrical. The external leaflet was associated typically with a variable amount of fibrillar material, but no distinct membrane-associated layer could be demonstrated in a variety of epithelial tissues. In addition, a distinct surface layer was not demonstrated by fixation with potassium permanganate solution containing lan-
thanum nitrate and nothing comparable to the dense layer of lanthanum-staining mate-
rial described by Lesseps was found associated with the external leaflet of the plasma membrane.

In glioblastoma multiforme, the particulate ruthenium red reaction product occurred with equal intensity in the narrow channels between adjacent cell processes and in the expanded intercellular spaces. It seems unlikely that the reaction depended simply upon the non-specific reactivity of absorbing surfaces. The reaction product which appeared closely associated with extracellular microfibrils was not directly asso-
ciated with plasma membranes, although there was a tendency for microfibrils to ac-
cumulate immediately adjacent to plasma membranes (Fig. 1 bottom). Similar accumula-
tions of microfibrils along cell surfaces have been found in other tissues, also.

These data concerning the submicroscopic distribution of ruthenium red in glioblastoma multiforme may be applicable to the inter-
pretation of the ruthenium red reaction in normal brain, where apposing cells are only about 200 A apart and it is more difficult to identify the glycocalyx or cell coat of indi-
vidual cells. These data suggest that in nor-

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Fig. 4. Potassium permanganate-lanthanum nitrate preparation. The coarse particulate reaction prod-
uct, seen in the extracellular space (*), is not associated exclusively with tumor cell plasma membranes
(arrows). Unstained, × 54,000.
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Summary

Specimens of glioblastoma multiforme were obtained at craniotomy by excisional biopsy. They were fixed in solutions containing ruthenium red, sectioned, and examined under an electron microscope. The dense, particulate, ruthenium red reaction product was found to be distributed selectively in the extracellular spaces of the tumor. It appeared to be associated closely with a variety of non-collagenous microfibrils demonstrated in other specimens fixed by immersion in glutaraldehyde-OsO₄ or in OsO₄ only. The ruthenium red reaction product did not appear to involve differentially the plasma membranes or substances immediately associated with plasma membranes.

References