Induction of Ocular Tumor by Nickel Subsulfide in the Japanese Common Newt, *Cynops pyrrhogaster*

Mitsumasa Okamoto
Institute of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464, Japan

ABSTRACT

Chemically induced ocular tumors in amphibians have not been reported previously. In the present study, nickel subsulfide (Ni$_3$S$_2$) was administered to lentectomized Japanese common newts, *Cynops pyrrhogaster*, by a single injection into the posterior chamber of the right eye (40–100 μg Ni$_3$S$_2$/newt). Control newts received a similar injection of vehicle. Malignant melanoma-like tumors developed in the injected eyes of seven of eight Ni$_3$S$_2$-treated newts at 9 months (versus none of six controls). Tumor cells occupied the entire globe and invaded the surrounding tissues. At 11 months melanoma-like cells in treated eyes exhibited by electron microscopy deeply invaginated nuclei and various degrees of pigmentation. Some cells even contained crystalline Ni$_3$S$_2$ in the cytoplasm at 11 months. Mitotic figures were rare in treated eyes at 9 months but were often found in them at 3 months. In one of the seven tumor-bearing eyes at 9 months, a prominent metaplastic cartilage was observed within the globe. A regenerated lens was noted at 9 months in a Ni$_3$S$_2$-treated eye which escaped tumor induction. The site of tumor origin was assumed to be the iris, because the aberrantly proliferating cell population was predominantly found in the root of the iris in treated eyes at 3 months.

INTRODUCTION

There are few reports of spontaneous (1) or chemically induced (2) tumors in urodeles compared to other animals. In normal and lentectomized eyes in urodeles, spontaneous tumors have not been recorded, and attempts to induce tumors with chemical carcinogens have been unsuccessful. Eguchi and Watanabe (3) inserted crystals of N-methyl-N'-nitro-N-nitrosoguanidine, a potent carcinogen, into lentectomized newt eyes and obtained supernumerary lenses from parts of the marginal iris other than the dorsal iris, cells of which have a lens-forming potential. However, they observed no tumor production.

An attempt was made to induce ocular tumors in newts with the chemical carcinogen, nickel subsulfide (Ni$_3$S$_2$). The present study shows that this nickel compound is carcinogenic to lentectomized eyes in the newt, which has been suggested to be resistant to most carcinogens (4).

MATERIALS AND METHODS

Animals. Adult Japanese newts, *Cynops pyrrhogaster*, collected in Tagarasu, Fukui Prefecture, Japan, were used. They were kept in an aquarium at 21 ± 2°C and fed on bovine liver chips.

Test Compound. Ni$_3$S$_2$ (in crystalline form; average particle size, <10 μm) together with an analysis table, was kindly provided by INCO, Ltd., Toronto, Ontario, Canada.

Lentectomy and Intraocular Injection of Carcinogen. Animals were anesthetized with 0.1% aqueous solution of MS222 (Sankyo, Inc., Tokyo, Japan). Each animal was transferred to an operation dish filled with Kesseljak's saline (103 mM NaCl, 0.9 mM CaCl$_2$, 10 mM KCl, and 1.2 mM NaHCO$_3$), and the right lens was extirpated with a pair of forceps through a slit made on the cornea. After the newt was removed from the operation dish, an injection into the posterior chamber of the right eye was performed with a glass micropipet. Each newt in the control group received an intraocular injection of 2–3 μl of sterile 0.6% NaCl solution or eye dropper oil. In an earlier experiment, the eye dropper oil used in clinical ophthalmology, which is composed of an equal volume of 1% aluminum monostearate and liquid paraffin, was used as the Ni$_3$S$_2$ vehicle, but in a later experiment the oil was replaced by 0.6% NaCl solution, because droplets sometimes delayed the growth of the regenerated lens in control. Each newt in the experimental group received a similar intraocular injection of the suspension containing 40–100 μg of Ni$_3$S$_2$.

The suspension of Ni$_3$S$_2$ in sterile NaCl solution (20–50 mg Ni$_3$S$_2$/ml) was continuously agitated with a magnetic stirring apparatus to ensure that constant amounts of Ni$_3$S$_2$ were aspirated into the micropipet. Despite this precaution, the Ni$_3$S$_2$ dosage could not be precisely controlled because of partial sedimentation of Ni$_3$S$_2$ particles within the lumen of the pipet. The control and Ni$_3$S$_2$-treated animals were kept on a humidified urethane mat in the container for 2 days and then in water at a temperature of 21 ± 2°C.

Histological Examinations. For light microscopic studies, Ni$_3$S$_2$-treated and control animals were decapitated at 3 and 9 months after the injection. The isolated heads were fixed with Bouin's fluid, decalified in 5% trichloroacetic acid in 70% ethanol at 4°C, and then dehydrated and embedded in TISSUEMAT (Fisher Scientific Co.). Serially cut sections 10 μm thick were stained by Mallory's method. For electron microscopy, 5 Ni$_3$S$_2$-treated animals were decapitated at 11 months after the injection. The isolated dorsal heads were fixed at 4°C overnight in 6% glutaraldehyde in Hanks' solution diluted to 80% of original concentration (pH 7.2–7.4; 109 mM NaCl, 4.3 mM KCl, 1.0 mM CaCl$_2$, 0.6 mM MgSO$_4$•7H$_2$O, 0.4 mM KH$_2$PO$_4$, 0.3 mM Na$_3$HP0$_4$, 2H$_2$O, 3.6 mM NaHCO$_3$, and 4.4 mM glucose). They were dissected into two halves and postfixed in cold 1% osmium tetroxide in the diluted Hanks' solution for 1 h. The tissue fragments were then block stained with 0.5% aqueous uranyl acetate solution for 1 h, washed, dehydrated in graded series of ethanol, immersed in propylene oxide, and embedded in Epon. Sections were cut with a diamond knife with a Reichert ultramicrotome, collected on carbon-coated grids, stained with uranyl acetate and lead citrate, and then examined under a JEOL 100C electron microscope at 80 kV.

RESULTS

Macroscopic Changes after Intraocular Injection of Ni$_3$S$_2$. From 1 week after the intraocular injection, vehicle control, Ni$_3$S$_2$-treated (right), and nonoperated (left) eyes were examined externally at weekly or biweekly intervals for up to 3 months. The cornea of the Ni$_3$S$_2$-treated eyes gradually became opaque within 1 week and then increased its opacity with time. For this reason, it was difficult to follow the intraocular changes externally. The cornea in the vehicle control eyes showed similar changes in the earlier stage but gradually recovered its transparency in all cases. Regenerated lens was found in all control eyes. Nonoperated (left) eyes did not show any changes throughout the experiments. In the controls, the wound opening of the cornea made by scalpel in lentectomy was sealed rapidly. However, the corneal wound in the Ni$_3$S$_2$-treated eyes did not heal in many cases even after 9 months.

Histology of Ni$_3$S$_2$-treated Eyes at 3 Months after Intraocular Injection. In treated eyes at 3 months, a cell population consisting of disarranged and aberrantly proliferating pigment cells (Fig. 1A) was encountered in the iris in 19 of 28 treated eyes. Mitotic figures were often noted among these cell populations.
Thickening of the corneal epithelium, composed of round nuclei, indistinct nucleoli, and scanty reddish cytoplasm after Mallory's stain. These cells could not be identified as neuroblasts, although similar cells could be found in the control neural retina.

Transmission electron microscopy performed on ocular tumors fixed at 11 months after the Ni$_3$S$_2$ injection revealed that tumor cells had various sizes and shapes and many of them possessed numerous microprojections on their surfaces (Fig. 3A). The nuclei of the cells were irregular and showed deep invaginations in some places at the nuclear membrane (Fig. 3, A and B). The nuclei were usually located on the eccentric region in the cell. A few distinct nucleoli were easily observed within the nuclei (Fig. 3A). Numerous electron-dense pigment granules were found in the cytoplasm of the melanoma-like cells (Fig. 3, A and C), but the extent of the pigmentation was different from cell to cell. Free-floating pigment granules could not be found in the intercellular space in the lesion. Crystalline and melanin-like inclusions were occasionally found in some melanoma-like cells (Fig. 3B). The cytoplasm contained a small amount of rough endoplasmic reticulum, Golgi complexes, and swollen mitochondria. Lamellar bodies (Fig. 3D) were often found but lysosomes and phagosomes which characterize the macrophage were poor in these cells. Acid-fast bacteria, the presence of which is reported in infectious granuloma cells (6), were not found in the cells even in detailed observation. In the lesions, some cells that differ from melanoma-like cells were observed. Although it was difficult to identify them, they showed characteristics similar to those of leukocytes.

The sclera became thin and loose in many places, from which tumor cells invaded surrounding tissues such as connective and muscle tissues (Fig. 4A). In some cases, infiltrated malignant tumor cells had direct contact with mucous glands in the cutis (Fig. 4B). Since the entire globe was filled with tumor cells, and ocular tissues such as iris, retina, and choroid had been destroyed, the sites of tumor origin could not be determined in the treated eyes at 9 months. However, it was suggested that aberrantly proliferating cells observed in the iris at 3 months (Fig. 1A) may be the sites of tumor origin.

Other Lesions. The lens was regenerated in an Ni$_3$S$_2$-treated eye in which no tumor was observed. In one case (Fig. 5A), prominent metaplastic cartilage was located between the tumor tissue and sclera. A mitotic figure was found in this cartilage (Fig. 5B). In control eyes, cartilage was never found within the eye chamber.

**DISCUSSION**

The present study demonstrated that melanoma-like ocular tumors were induced with high incidence in lentectomized newt
OCULAR TUMOR IN JAPANESE NEWTS INDUCED BY Ni$_3$S$_2$

Fig. 2. A, Ni$_3$S$_2$-treated eye occupied by tumor cells at 9 months. ce, corneal epithelium; cs, corneal stroma; sc, sclera; mg, mucous gland. Mallory stain, x 33. B, high magnification of part of the tumor shown in A. Mallory stain, x 250. C, remnants of neural retina in the tumor at 9 months. ar, neural retina. Mallory stain, x 50. D, eye with regenerated lens in vehicle control at 9 months. ce, corneal epithelium; cs, corneal stroma; i, iris; l, lens; ar, neural retina; ch, choroid; sc, sclera. Mallory stain, x 22.

Fig. 3. A, electron micrograph of melanoma-like cells in Ni$_3$S$_2$-treated eye at 11 months. N, nucleus; pg, pigment granule; mp, microprojection. x 2,900. B, electron micrograph of melanoma-like cells in Ni$_3$S$_2$-treated eye at 11 months, showing crystalline Ni$_3$S$_2$ (arrow). N, nucleus. x 5,000. C, high magnification of part of the numerous pigment granules shown in A. pg, pigment granule. x 23,200. D, electron micrograph of melanoma-like cells at 11 months. The cytoplasm contains a well-developed lamellar body. x 10,000.
eyes by intraocular injection of nickel subsulfide, Ni$_3$S$_2$. This study is the first report of chemical induction of tumors in the Japanese common newt, C. pyrrhogaster, and in the eyes of amphibians.

The carcinogenic properties of nickel compounds have been reviewed by Sunderman (7), who mentions that Ni$_3$S$_2$ is the most potent carcinogen among the numerous nickel compounds that have been tested. Albert et al. (8) induced intraocular malignant melanomas, retinoblastomas, and gliomas of the optic nerve by injection of crystalline Ni$_3$S$_2$ into rat eyes. In the present study, we cannot exclude the possibility that the lesions observed in Ni$_3$S$_2$-treated eyes at 9 months were not caused by melanomas, because the cytochemical identification of melanin or the measurement of the tyrosinase activity and the biosynthetic activity of melanin were not performed in the pigment granules found in the melanoma-like cells.

Dawe (9) claimed in his review article that many of the amphibian diseases thus far reported as "neoplasms" were not true neoplasms but infectious granulomas. In the present study, bacteria were not found in the melanoma-like cells. Although the numerous microprojections and a few phagosomes observed in the cells in the present study partly suggested that these were not melanotic melanoma cells but may be macrophages which actively phagocytized the growing tips of the rod outer segments according to the light and shade reaction. Hollyfield (10) demonstrated that amphibian retinal pigment epithelium in various developmental stages actively phagocytosed the polystyrene beads which had been injected into the space bounded by the neural retina and pigment epithelium. It is also known that iris and retinal pigment epithelia develop from the same progenitor cells in the embryonic stage and are connected at the ora serrata. Thus, it is reasonable to assume that iris pigmented epithelial cells have an active phagocytic activity. The fact that the melanoma-like tumors were induced with a high incidence in Ni$_3$S$_2$-treated eyes of newts in the present study may be partly explained by the action of active phagocytic activities of iris pigmented cells.

Crystalline Ni$_3$S$_2$ observed by electron microscopy supported this idea.

$N$-Methyl-$N'$-nitro-$N$-nitrosoguanidine has well known mutagenic (11) and carcinogenic (12) activities. As to the induction of tumors in lentectomized newt eyes, Ni$_3$S$_2$ in the present study showed a carcinogenic effect, while $N$-methyl-$N'$-nitro-$N$-nitrosoguanidine did not show such properties in the previous study (3). Nishimura and Umeda (13) showed the induction of chromosomal aberrations in the cultured mammalian cells treated by nickel compounds such as potassium cyanonickelate and nickel sulfide. From in vivo (14) and in vitro (15) studies, it has been shown that nickel compounds induce DNA breaks. It therefore seems likely that Ni$_3$S$_2$ acts as a potent mutagen on the pigmented cells in the newt eye and binds to nuclear DNA to make tumor cells. The newt is advantageous for the analysis of chromosomal aberrations and changes on the DNA level, since the genome of the C. pyrrhogaster is only 12 (16), whereas the DNA content per nucleus in newts is usually 10 times that of many other vertebrates (17).

An experiment revealing that lens regeneration was inhibited by 60–70% in the Ni$_3$S$_2$-treated eyes fixed at 25 days and 3 months after lentectomy will be reported elsewhere. The molecular analysis of the mode of action of Ni$_3$S$_2$ in the present system can be expected to provide useful information for accessing the mechanism of carcinogenesis and regeneration in urodeles that seem to be resistant to most carcinogens and that have high regenerative capacities.

**ACKNOWLEDGMENTS**

The author thanks Dr. G. Eguchi, Dr. M. Matsuyama, and Dr. T. Ogiu for their kind consultation in interpreting the histological slides.

1 M. Okamoto. Inhibition of lens regeneration by chemical carcinogen, nickel subsulfide in the Japanese common newt, Cynops pyrrhogaster, manuscript in preparation.
Gratitude is also expressed to Dr. T. Ogiu and Dr. M. Matsuyma for their critical reading of the manuscript and valuable discussions. The author is indebted to Dr. M. Araki and Dr. A. Masuda for their kind assistance in the course of this study.

REFERENCES

Induction of Ocular Tumor by Nickel Subsulfide in the Japanese Common Newt, *Cynops pyrrhogaster*

Mitumasa Okamoto


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/19/5213

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.