Malignant Transformation of Hamster Embryonic Cells by 4-Hydroxyaminoquinoline N-oxide in Tissue Culture*

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In the present study, hamster embryonic cells were successfully transformed by 4-hydroxyaminoquinoline N-oxide (4HAQO), an active metabolite of 4-nitroquinoline N-oxide (4NQO). The transformed cells were characterized by randomly oriented arrangement of fusiform cells and continuous proliferation *in vitro* over 200 days. The transformed cells, cultured for 56 days *in vitro* and transplanted into newborn and adult hamsters, produced tumor and finally killed the animals. Histologically, the tumor was identified as fibrosarcoma.

In untreated cultures, the cells revealed a limited growth and failed to produce tumor in animals.

As compared to carcinogenesis by virus, that produced by chemical or physical agents has a disadvantage that an *in vitro* experiment is hardly applied. Since several years, a research group† has attempted to establish experimental systems of *in vitro* carcinogenesis using several combinations such as dimethylaminoazobenzene (DAB) or 3'-methyl DAB on rat liver cells,¹⁻⁷ actinomycin on mouse embryonic cells, and 4-nitroquinoline N-oxide (4NQO) on tissue culture of hamster skin. The present paper deals with successful malignant transformation of hamster embryonic cells by 4-hydroxyaminoquinoline N-oxide (4HAQO), which is an active derivative of 4NQO.

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MATERIALS AND METHODS

Cell culture. Embryonic cells of golden syrian hamster were used as normal cells. Minced whole embryos, of which the head, lungs and heart had been removed for other experiments, were cultured by the method of explant culture. A part of the harvest was transferred on the 5th day, and the remainder was preserved in liquid air as a batch of cell source. Carcinogen was applied in the secondary culture.

The standard medium used was autoclaved Eagle's minimum essential medium supplemented with 1.0 mM pyruvate, 0.2 mM serine, 1.0 mg/l biotine and 10% heat-inactivated bovine serum. For subcultures, confluent cultures were digested with 0.025% pronase (Kaken Kagaku Co.) solution. The cells were inoculated into TD-40 flasks or flat-sided culture vessels at a concentration of 10^5 cells/ml. Change of medium was made twice per week.

In the experiments of colony formation, albumin medium was employed following the advice of Prof. I. Yamane of our Institute.⁸ The albumin medium consisted of 0.75% bovine albumin fraction V (Armour Pharma. Co.), 0.1% Bacto-peptone (Difco Lab.) and 20% heat-inactivated bovine serum in the standard medium, which was sterilized by Millipore filtration. Cells for cloning were seeded in 50 mm Petri dishes without feeder layer at concentrations of 10^2 to 10^5 cells per dish (25 to 25,000 cells per ml).

Treatment with carcinogen. The carcinogen used in this study was 4-hydroxy-aminoquinoline N-oxide—HCl (4HAQO), which was kindly supplied by Dr. M. Yamada of National Institute of Health, Tokyo. Special attention was paid in preparation and treatment with the carcinogen, because the compound is very unstable in the culture condition. The working solution of carcinogen was prepared by mixing 9 parts of distilled water with one part of 10^{-2} M ethanol solution of the compound. This solution was sterilized by Millipore filtration. Application of carcinogen was done as follows. Working solution was added directly on cell sheets, after culture medium was discarded or gathered to the bottom of vessels. Within 30 seconds, the cultures were refed with fresh medium. The final concentration of the carcinogen was 10^{-5} M (2.1 γ /ml). The treatment was repeated every other day, and at the end of the whole course of the treatment, the cultures were washed three times with phosphate buffer solution and once with standard medium.

Transplantation experiments. For assay of tumorigenicity, the cultured cells were transplanted into the cheek pouch or the subcutaneous tissue of adult golden hamsters, or the subcutaneous tissue of newborn animals within 24 hours after birth. The animals had received no conditioning treatment such as injection of cortisone or x-ray irradiation. Development of tumor was examined at weekly intervals by palpation or measurement. Histological study of tumor

and organs was carried out in the usual manner.

RESULTS

Growth and morphology of non-treated culture

In the control experiment, 4 substrains of non-treated culture were maintained for more than 200 days. During the first 20 days, the cells of controls grew actively and could be transferred at 4 or 5 days intervals. The cells at this period appeared to be fibroblastic, showing an oriented arrangement of monolayer (Fig. 1). After 30 days in culture, however, a decrease in proliferation rate was observed as indicated by less harvest of cells and longer interval of transfer. Cultures were hardly transferred later than 50 days *in vitro*. Such a decrease in growth was attended with changes of cell morphology. The cells after 30 days *in vitro* had large, flat and granulated cytoplasm, as shown in Fig. 2. Although the controls had been maintained for over 200 days, there were no signs of establishment as a permanent cell line. Such a limited growth of embryonic hamster cells was repeatedly noted with the cells which were obtained by thawing the cell batch in liquid air.

Morphological transformation of carcinogen-treated cells

The carcinogen was introduced in 2-day-old secondary cultures (7 days *in vitro*) and thereafter the treatments were continued for 12 days at 1 day intervals. This schedule is abbreviated as (7-12). On the 20th day *in vitro*, the cultures were transferred and divided into following two sublines; the one was maintained in carcinogen-free medium, and the other was submitted to further treatments for 14 days. These were designated as HA-1 and HA-2 lines, respectively. Finally, total time and schedules of treatments were 6 times (7-12) in HA-1 line and 13 times (7-12-5-14-) in HA-2 lines.

The effects of carcinogen appeared 2 or 3 days after the treatment and were characterized by cell necrosis and a criss-crossed arrangement of fusiform cells in parts of the cultures (Fig. 3). The fusiform cells grew even after the cell sheet was confluent. Thus, the cells piled up and formed dense felt-like layer, while parts of the culture revealed a monolayer growth of non-transformed cells. After the transfer, there was a time lag to appearance of the transformed foci (Fig. 4), which grew over the background of non-transformed cells and showed continuous proliferation. Thus the carcinogen-treated cultures could pass the crisis of growth as seen in the controls and have been maintained stably by serial transfers *in vitro*. Such a typical course of transformation was noted in both HA-1 and HA-2 lines. The doubling time, estimated in 223 days *in vitro*, was 14.3 hours in HA-2 line. Chromosomes of the transformed cells were aneuploidic and distributed widely.

Colonial growth of the cells served to elucidate the morphological transforma-

tion. As shown in Fig. 5, the colonies developed from the treated culture exhibited a pattern of randomly oriented growth. Besides such dense colonies, colonies which were formed by oriented fibroblasts or by large and flat cells were also noted. The plating efficiency of the treated culture was very low in the early stage of transformation, *i.e.*, when 10,000 cells were seeded per dish in 34 days *in vitro*, about 0.4% of cells could form colonies. However, serial cultivations for more than 100 days resulted in such a marked increase in the efficiency as large as 30% or so.

Transplantation experiments of treated cultures and the controls

As summarized in Tables 1 and 2, HA-1 and HA-2 cells successfully gave rise to tumors in newborn and adult hamsters. HA-1 cells of 56 days *in vitro* (7-12-37) produced progressively growing tumors in newborn hamsters after a long latent period of 4 months, while in adult hamsters small nodules were formed and regressed. After 77 days *in vitro* (7-12-58), HA-1 cells could grow in adult hamsters without any latent period of growth. HA-2 cells were also successfully transplanted on 65th day (7-12-5-14-27) both in adult and newborn hamsters, without latent period or initial regression. The animals died of tumor with metastasis (Fig. 6). Histologically, the tumors were diagnosed as fibrosarcoma, while in some areas they accompanied a few eosinophilic leukocytes and Langhans' type giant cells (Fig. 7). The re-culture of these tumors yielded typical trans-

TABLE 1. *Transplantation of HA-1 cells into hamsters*

Culture day	Age	Site	No. of cells transplanted	Results†	Survival days
5G. 56d. (49d.)*	Newborn	sc†	1.0×10^6	3/3§	175, 182, 205
	Adult	sc	"	0/2'	
	"	ch.p	"	0/2'	
7G. 77d. (70d.)	Adult	sc	1.0×10^6	1/1	86
	"	"	5.0×10^6	1/1	101
	"	ch.p	1.0×10^6	2/2	47
	"	"	5.0×10^6	2/2	Sacrificed
10G. 89d. (82d.)	Adult	ch.p	3.0×10^6	4/4	Sacrificed
	"	"	1.0×10^6	6/6	"
	"	"	3.0×10^5	6/6	"
	"	"	1.0×10^5	6/6	"
	"	"	3.0×10^4	1/2	"
14G. 117d. (110d.)	Adult	ip	3.0×10^6	2/2	44, 48

* Numbers in parentheses show culture days after the first treatment.

† sc, subcutaneous; ch.p, cheek pouch; ip, intraperitoneal.

‡ Number of animals or cheek pouches with tumor growth/number of examined.

§ Tumors were found 4 months after the transplantation.

' Small and hard nodules were formed at the site of transplantation, and regressed after one month.

TABLE 2. *Transplantation of HA-2 cells into hamsters*

Culture days	Age	Site	No. of cells transplanted	Results†	Survival days
4G. 65d. (58d)*	Newborn	sc†	1.0×10^6	6/6	74, 80, 80, 91, 96, 119
	Adult	sc	5.0×10^6	1/1	91
	"	ch.p	5.0×10^6	2/2	91
	"	"	3.0×10^6	2/2	91
9G. 101d. (94d.)	Adult	ch.p	3.0×10^6	2/2	Sacrificed
	"	"	1.0×10^6	2/2	"
	"	"	3.0×10^5	6/6	"
	"	"	1.0×10^5	4/6	"
	"	"	3.0×10^4	2/4	"

* Numbers in parentheses show culture days after the first treatment.

† sc, subcutaneous; ch. p, cheek pouch.

‡ Number of animals or cheek pouches with tumor growth/number of examined.

TABLE 3. *Transplantation of control cells*

Culture days*	Age	Site†	No. of cells transplanted	Results‡
19d.	Adult	ch.p	2.0×10^6	0/2
26d.	Adult	ch.p	1.0×10^6	0/2
26d.	Newborn	sc.	1.0×10^6	0/6
33d.	Adult	ch.p	5.0×10^6	0/2
138d.	Adult	ch.p	1.0×10^6	0/1

* Culture days do not include the time of preservation in liquid air.

† sc, subcutaneous; ch. p, cheek pouch.

‡ Number of cheek pouches or animals with tumor growth/number of examined.

formed cells and colonies.

Table 3 shows the data of transplantation in the controls. Although the controls gave rise to small and soft nodules in the cheek pouch of the hamster for a while, these regressed entirely in the second week of transplantation. Histologically, the nodules were entirely devoid of any malignant features and consisted of necrotic foci and reaction of host cells (Fig. 8). No tumors were produced by subcutaneous transplantations into newborn animals.

COMMENTS AND CONCLUSION

Establishment of an *in vitro* system would contribute much to studying the mechanism of carcinogenesis. Since Earle's experiments in 1943,⁸ considerable efforts have been made for this purpose. Recently, Berwald and Sachs reported that hamster embryonic cells could be transformed *in vitro* by carcinogenic hydrocarbons.⁹

In the present study, hamster embryonic cells were successfully transformed to tumor cells by treatments with 4-hydroxyaminoquinoline N-oxide (4HAQO).

This compound is supposed to be an active metabolite of 4-nitroquinoline N-oxide (4NQO).¹⁰⁻¹⁴ The transformed cells were characterized by randomly oriented arrangement of fusiform cells and their continuous proliferation *in vitro* over 200 days. Transplantation of these cells gave rise to tumors in newborn and adult hamsters after 56 days *in vitro*, and finally killed the animals. The tumor was diagnosed histologically as fibrosarcoma. As compared to application of these carcinogens in animals,¹⁰⁻¹³ malignant transformation *in vitro* was recognized in such a short time as 49 days after the treatment (56 days in total culture days).

In untreated cultures, no such transformed cells were found. The controls in the early period of culture revealed a monolayer growth of fibroblastic cells, which was later followed by nonproliferating state of large, flat and granulated cells. The cells of controls failed to grow when inoculated into newborn and adult animals. Such a limited life span of normal hamster cells was in accord with observations by Todaro,^{15,16} Berwald⁹ and Yamane (personal communication). Thus the experimental system used in this study provides an advantage in that carcinogenic effect of an agent may develop without any appreciable spontaneous cell transformation.

Besides 4 HAQO, carcinogens such as 4 NQO and 6-chloro 4 NQO were examined in cultures under the identical conditions. These compounds also effected malignant transformation of hamster embryonic cells.¹⁷ The details of the experiments will be reported elsewhere.

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Legends

- Fig. 1. Oriented arrangement of fibroblastic cells of the controls in 19 days *in vitro* (5 transfer generations). Wright-Giemsa stain, 4×10 .
- Fig. 2. Late picture of the controls. Note the large, flat and granulated cells. On the 38th day *in vitro* (5 transfer generation). Phase-contrast, 10×10 .
- Fig. 3. Change of cell arrangement appeared 3 days after the treatment with carcinogen. Phase-contrast, 10×10 .
- Fig. 4. Criss-crossed arrangement of the cells of transformed foci, but the background appeared as in the controls. HA-1 cells, 32 days *in vitro* (7-12-13). Phase-contrast, 10×10 .
- Fig. 5. Colony of the transformed cells, which was obtained by seeding 1.0×10^4 cells of HA-1 in a dish on the 34th day *in vitro* (7-12-15). Eleven days incubation. Note that cells distributed randomly and formed a dense colony. Wright-Giemsa stain, 1.2×10 .
- Fig. 6. Animal which died of tumor on the 74th day of subcutaneous transplantation of 1.0×10^6 of HA-1 cells into a newborn hamster. Note the large tumors which developed along the needle of syringe at the transplantation.
- Fig. 7. Histological picture of the tumor in Fig. 6. The diagnosis was fibrosarcoma. Hematoxylin-eosin stain, 4×10 .
- Fig. 8. Histological picture of the tumor of control, which was resected 7 days after transplantation into the cheek pouch of a hamster. Note that the tumor consisted of necrotic foci and reaction of host cells. Hematoxylin-eosin stain, 4×10 .