Further Studies on the Therapeutic Effect of Indomethacin on Esophageal Tumors

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The effect of treatment with the nonsteroid anti-inflammatory drug indomethacin, was investigated in 250 3-month-old C57Bl female mice following induction of esophageal tumors with diethylnitrosamine (DEN). Already 3 months after DEN treatment, many esophageal tumors were committed to occur: despite discontinued DEN treatment, the number of tumors had increased significantly at 6 or 9 months of observation. Following 3 and 4 months of DEN administration, the development of many already committed esophageal tumors was significantly abrogated by treating the mice with indomethacin.

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THE OBSERVATION THAT ANIMAL AND HUMAN TUMORS contain high concentrations of prostaglandins (PG)[1–4] has prompted several investigators[2,5–9] to challenge tumor growth with drugs known to inhibit the endogenous prostaglandin biosynthesis. The drugs tested have been the so-called nonsteroid anti-inflammatory drugs (NSAID). Indomethacin, a NSAID, has lately been demonstrated to play a role in the control of neoplastic cellular proliferation,[2] in the immunologic reaction of the host,[1,6,10,11] and in the regression of PG-promoted tumor growth.

In a previous experiment,[7] the antitumoral activity of indomethacin on experimentally induced esophageal tumors in the mouse was investigated. The results demonstrated that the number of diethylnitrosamine (DEN)-induced esophageal tumors was significantly reduced when indomethacin was given together with that carcinogen from the onset of the test. Indomethacin was also demonstrated to exert an inhibitory effect on the appearance of additional esophageal tumors, when given 4 months after the carcinogen administration.

In the current work, further information is provided on the inhibitory activity of delayed indomethacin administration on the development of additional esophageal tumors in mice.

Materials and Methods

A total of 250 three-month-old C57Bl female mice were used. The animals were bred in our laboratory according to the guidelines of the Department of Pathology, Karolinska institute. Diethylnitrosamine (DEN; Merck, Sweden) was given in drinking water at a concentration of 0.4 mg/1000 ml drinking water 3 days a week for 3 or 4 months (see below). For this purpose, 32 mg indomethacin from a stock solution (Sigma Chemicals, USA) was dissolved in 10 ml abs. alc. From this, 2 ml was mixed in 4000 ml fresh water.

DEN or indomethacin was given according to the following scheme. All experiments were initiated simultaneously.

Experiment 1: A total of 105 mice were studied; 26 received DEN for 3 months, autopsy was performed immediately afterwards. Sixty-one mice were also treated for 3 months, but allowed to live 3 additional months with water *ad lib.* The animals were thus killed 6 months after initiation of DEN treatment. A third group of animals, comprising 18 mice, received DEN for 3 months and thereafter indomethacin in drinking water for 3 months. They were killed 6 months after initiation of DEN treatment.

Experiment 2: Thirty-seven mice were treated as follows: 19 received DEN for 3 months and for the following 6 months water *ad lib.* These animals were killed 9 months after initiation of DEN treatment. A second group of 18 mice received DEN for 3 months, followed by 6 months treatment with indomethacin in drinking water. These animals were also killed 9 months after start of DEN treatment.

Experiment 3: In this experiment, 108 mice were used: 39 received DEN for 4 months (autopsy was performed immediately after treatment). Thirty-five animals also received DEN for 4 months, but they were allowed to live 3 additional months with water *ad lib.* A third group of 34 mice received DEN for 4 months, followed by indo-

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TABLE 1. The Tumor Index in 105 C57Bl Mice Treated 3 Months With DEN

Treatment	No. of animals*	Tumor index†
DEN 3 days/week	26	0.70 SD ± 0.2
3 mo DEN 3 days/wk + 3 mo H_2O	61	5.01 SD ± 0.85
3 mo DEN 3 days/wk + 6 mo H_2)	19	2.85 SD + 1.25

* Twenty-six were autopsied immediately after, 61 were allowed to live 3 additional months on drinking water *ad lib,* and the remaining 18 with indomethacin added to the drinking water.
† The number of tumors/cm of esophageal mucosa.
DEN: diethylnitrosamine.

methacin in drinking water for three additional months. At the end of treatment the animals were killed.

The animals were killed by halotane anaesthesia. After thoracolaparotomy, the esophagus was resected in all its length, opened wide on a Millipore filter and fixed in methanol ascetic acid. After fixation, the preparations were studied in a dissection microscope by transillumination according to a previously described method.[12] The number of tumors in each esophagus was noted as well as length of resected esophagus. The ratio between the number of tumors and the length of esophagus (in cm), *i.e.,* the "tumor index," was recorded in each animal. To study the gross anatomy of the tumors, the fixed preparations were also studied by direct illumination in the same microscope.[8]

Results

In all animals, esophageal tumors ranging from 1 mm to 5 mm were found. The tumors were irregular in shape with either smooth cobblestone or cerebroid surface. Ulcerations were not found. No apparent differences in size or configuration of the tumors between DEN-treated controls and DEN/Indomethacin-treated mice could be observed. The difference found was in the tumor index in the various experiment groups.

Experiment 1: Table 1 shows that animals treated with DEN for 3 months (and killed immediately at completion of treatment) had a mean of 0.7 tumors/cm, while mice receiving the same treatment but allowed to live additional 3 months, had a mean of 5.01 tumors/cm. The difference was significant ($P < 0.001$). The third group also received DEN for 3 months, but they were given indomethacin in drinking water. These animals demonstrated a significantly lower ($P < 0.01$) number of tumors than control, water-treated mice (2.85 tumors/cm).

TABLE 2. The Tumor Index in 37 C57Bl Mice Treated 3 Months With DEN and Allowed to Live 6 Additional Months

Treatment	No. of animals*	Tumor index†
3 months DEN + 6 mo H_2O	19	4.79 SD ± 0.85
3 months DEN + 6 mo indomethacin	18	3.78 SD ± 0.90

* 19 on drinking water *ad lib,* and 18 having indomethacin added to the water.
† The number of tumors/cm of esophageal mucosa.
DEN: diethylnitrosamine.

TABLE 3. The Tumor Index in 37 C57Bl Mice Treated 4 Months With DEN and Allowed to Live an Additional 3 Months

Treatment	No. of animals*	Tumor index†
4 mo DEN	39	1.24 SD ± 0.60
4 mo DEN + 3 mo H_2O	35	5.23 SD ± 1.06
4 mo DEN + 3 mo indomethacin	34	3.55 SD ± 1.02

* 35 on drinking water *ad lib,* and 34 having indomethacin added to the water.
† The number of tumors/cm of esophageal mucosa.
DEN: diethylnitrosamine.

Experiment 2: The results in Table 2 show that animals given DEN for 3 months, and allowed to live additional 6 months with water *ad lib,* had a mean of 4.79 tumors/cm. The difference between this group and those given Indomethacin for 6 months after 3 months DEN treatment was significant ($P < 0.05$).

Experiment 3: Table 3 demonstrates that mice receiving DEN for 4 months and autopsied immediately after treatment had a mean of 1.24 tumors/cm. This was significantly lower ($P < 0.001$) than for mice receiving DEN also for 4 months followed by water *ad lib* for 3 additional months before being killed (5.23 tumors/cm).

When mice receiving DEN for 4 months were given indomethacin in drinking water in the following 3 months (Table 3), the number of esophageal tumors was only 3.55 tumors/cm (*i.e.,* significantly reduced; $P < 0.01$).

Discussion

The results of the current work demonstrated that the administration of a relatively low dose of DEN (only 3 days a week for 3 months) resulted in a relatively low number of tumors when the mice were killed immediately after that treatment. When the animals were allowed to live for additional 3 months without further treatment, a significant increase in the tumor index was found. This suggests that clones of esophageal cells were already committed to tumor growth at an earlier stage, but remained undetected at 3 month's observation. These findings also suggest that the significant increase in tumor index when DEN administration was prolonged from 3 to 4 months may be apparent.

The above results seem to mitigate against the hypothesis that, by increasing the dose of carcinogen and pro-

longing the time of DEN administration, the number of esophageal tumors would increase. Experiments have been initiated aiming to establish the lowest dose and the shortest time of DEN treatment required to "program" about 5 esophageal tumors/cm of esophageal mucosa in our model.

After the DEN treatment was completed, the addition of indomethacin to drinking water 3 days a week was sufficient to arrest a significant amount of apparently already "programmed" esophageal tumors. The arrest in tumor development with indomethacin was not increased by prolonging the time of indomethacin administration from 3 to 6 months after DEN treatment. Even here, it appears of the greatest interest to establish the lowest dose and the shortest time of indomethacin treatment required to inhibit the further development of esophageal tumors in our model.

In light of the results of these experiments, it may be concluded that many DEN-induced tumors in the esophageal mucosa of mice are bound to occur despite the discontinuation of carcinogen administration. The further development of some esophageal "committed" tumors can apparently be abrogated by the postcarcinogen treatment with indomethacin. The mechanism of action of Indomethacin in the abrogation of experimentally induced tumors remains unclear. Some claim that the substance acts by inhibiting the endogenous synthesis of prostaglandin.[1,13] Prostaglandin levels have been reported to be increased animal and human tumors.[1–4] On the other hand, others[6,11] believe that indomethacin may be an immune potentiator which acts independently of the prostaglandin synthesis inhibition. Further studies are required to elucidate the true mechanism of action of indomethacin.

Nonsteroid anti-inflammatory drugs have recently been used in humans as a co-adjuvant treatment of another squamous cell tumor, namely squamous carcinoma of the uterine cervix, with promising results.[9] Perhaps, indomethacin should also be incorporated as a co-adjuvant in the treatment of squamous cell tumors of the human esophagus, in attempts to improve the overall low cure rate obtained so far for this tumor.

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