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HYPERTHERMIA AND RADIATION IN TREATMENT OF HUMAN SQUAMOUS CELL CARCINOMA OF CERVIX XENOGRAFTED IN ATHYMIC MICE

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Introduction

Cytological screening by the use of the Papanicolaou smear has significantly reduced the number of patients with advanced cervical cancers. Early detection, epidemiology, correct diagnosis and treatment of early stages of cervical lesions have also been effective in control and prevention of cervical cancers. But invasive carcinoma of the cervix is still an important aspect of the cervical cancer problem. The greatest enigma at this time is the etiology, or predisposing factors, i.e., role of male and role of herpes and papilloma viruses in the development of these lesions, which classifies it as a sexually transmissible disease.[1-4] This and other psychological and socio-economic factors might hinder some patients from seeking early detection and prevention. There are still hundreds of cases with advanced lesions reported in literature. Beyond stage II, radiation and chemotherapy are the only treatment and may not be well tolerated by the patient. Serious complications due to radiation therapy occur most frequently in advanced stages of this disease because of high doses of radiation which are required to accomplish tumor control.[5] It is, therefore, important to seek a broader selection of modes of treatment for this cancer, especially in advanced stages.

It is now well established that hyperthermia kills cells in a manner as predictable and repeatable as radiation and chemotherapy agents.[6] Unlike ionizing radiation, hyperthermia kills both hypoxic and well oxygenated cells.[7] Furthermore, neoplastic cells tend to be more sensitive to hyperthermia than do normal cells.[8] In addition, heat interacts with radiation and chemotherapeutic agents.[9-10] The role of prostaglandin, prostaglandin analogues, and prostaglandin inhibitors in the treatment of cancer is still controversial. Misonidazol (RO-07--582) has been shown to be an electron affinic hypoxic cell sensitizer.[11-13] It minimizes the effect of oxygen as a reason for failure in the radiation of hypoxic tumors.

We report here the effect of hyperthermia, radiation and chemotherapeutic agents, used alone or in combination, on human carcinoma of the cervix transplanted into athymic (nude) mice, which may offer a broader selection for the treatment of this lesion in human patients.

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Materials and Methods

Human squamous cell carcinomas of the cervix from patients admitted to St. Louis University Hospitals were used in these studies. Immediately after excision, the tumors were placed in sterile petri dishes. The solid parts of tumor were washed twice in sterile phosphate buffered saline (PBS), then were minced into very small particles, and a small amount of PBS was added. Small quantities (0.1ml) of this tumor mixture were injected through a 15 gauge needle into the left hind leg muscles of 6-8 week old female athymic (nude) mice having Balb/C genetic background. A small piece from each original tumor was processed for histological study. All mice were housed, four per cage, at 80°F in air-tight glass door cabinets protected by laminar air flow, and were given autoclaved chow and water. Most of these mice (70-80%) developed tumors 8-10 weeks after the tumor injections. Histologically, the tumors in mice resembled the original human tumors which were used for xenografting. When the tumors in mice reached about 1 cm in diametre, the mice were divided into groups for each planned treatment.

In the case of hyperthermia, the hind limbs of the mice having tumors were suspended in a constant temperature hot bath at 43°C for 40 minutes. After treatment, the mice were placed under a hot lamp (80°F) to prevent cold exposure. Heat treatments were given twice a week for a total of five treatments.

The radiation treatment was administered with a Siemens Gammatron S teletherapy unit (output 100 rads/min. at 80cm SSD). Auxiliary lead collimating blocks (total height 8 inches) having 1.5cm holes were used to confine the radiation field to the area of the tumor and to minimize the whole body dose. Five fractions of 1500 rads each were delivered over a three week period to the depth of 0.5cm using a single *an fass* port. The mice were treated individually to minimize the chance of a geographic miss. To stabilize, the mice were lightly anaesthetized with 20mg/kg of sodium thiamyll before radiation treatment.

In experiment 1, the mice having tumors were divided into three groups. Group I received heat treatment alone; Group II received heat treatment and radiation; Group III received radiation alone.

In experiment 2, the mice having tumors received 15mg misonidazole subcutaneously and were divided into three groups. Group I received misonidazole alone; Group II received misonidazole and heat treatment; Group III received misonidazole and radiation.

In experiment 3, the mice having tumors were treated with indomethacin (20mg/kg orally) or Ibuprofen (The Upjohn Co., 20mg/kg orally) and were divided into four groups. Group I received indomethacin alone; Group II received indomethacin and heat treatment; Group III received Ibuprofen alone; Group IV received Ibuprofen and heat treatment.

In experiment 4, 16, 16 dimethyl PGE_2 (The Upjohn Co., Kalamazoo, MI), 50ug/kg, was injected intraperitoneally, and the mice were divided into two groups. Group I received the prostaglandin analogue only, and Group II received prostaglandin and hyperthermia.

In experiment 5, the mice having tumors were treated with adriamycin (0.4mg/kg) and graded doses of radiation. Group I received 500 rads, Group II received 750 rads, Group III received 1,000 rads, and Group IV received 1,500 rads of radiation in each treatment. This experiment was designed to determine the dose of radiation which could be most effective for these treatments. In our previous studies,[5] adriamycin was found to be a very effective radiosensitizer.

Some mice having tumors were not treated and served as controls. In each experiment, mice received bi-weekly treatments for a total of five treatments and were observed for six months after the treatments. The tumors were measured and recorded every week by the same person. (16, 16 diamethyl PGE_2 and Ibuprofen were kindly supplied by Drs. Pike and Lammer of the Upjohn Co., Kalamazoo, MI).

Results

The results of these experiments are shown in tables. In experiment 1 (Table I), the tumors in mice treated with hyperthermia alone regressed continuously and disappeared seven weeks after the treatment. In another experiment where the tumors were somewhat larger (2.0 0.5cm), heat treatment was not so effective. The tumors in these mice continued to grow, although more slowly than those in untreated animals. The tumors in mice treated with hyperthermia and irradiation regressed continually and, except in one mouse, totally disappeared 8 weeks after the treatment. The tumor in this mouse, palpable at this time, disappeared completely 10 weeks after the treatment. The tumors in mice treated with radiation alone also showed regression, except in two mice which did not respond to the treatment. These two mice with large tumors (2.2cm and 1.9cm) died seven weeks after the treatment. Recurrence of tumors was not seen in any of the treated mice until six months after treatment. There was no evidence of radiation sickness in any of the mice.

In experiment 2 (Table II), the tumors in mice treated with misonidazole alone continued to grow. The rate of growth in these tumors was much slower than that in untreated mice. In the group of mice treated with misonidazole and hyperthermia, tumors regressed continually. Eight weeks after the treatment, only one mouse had a palpable tumor. The tumors in mice treated with misonidazole and irradiation showed regression except for one mouse which had a palpable tumor. This tumor also disappeared completely one week later. The effect of misonidazole was more pronounced when combined with radiation than when combined with hyperthermia.

In experiment 3 (Table II), the tumors treated with indomethacin or with Ibuprofen continued to grow. The growth of tumors in mice treated with indomethacin was somewhat slower than in the mice treated with Ibuprofen. The tumors of mice treated with indomethacin and hyperthermia or Ibuprofen and hyperthermia showed continuous regression. Tumors in all mice disappeared six weeks after treatment except for two mice (one from each group) which died four weeks after treatment. The tumors in these mice also showed relative regression. Some of the mice died during or shortly after heat treatment from dehydration or other causes. These mice were not included in the data presented in results. The mice treated with indomethacin and heat, due to some unknown cause, died six weeks after the treatment.

Because indomethacin and Ibuprofen are inhibitors of prostaglandins, an experiment using prostaglandin analogue 16, 16 dimethyl PGE_2 alone and in combination with hyperthermia was performed. The results of experiment 4 were very inconsistent. The tumors in some mice showed a slight regression during the treatment period, and afterward started to increase in size again. Other mice did not respond to this treatment at all.

In experiment 5 (Table III), the treatment with 1,500 rads was found to be most effective. As the radiation dose was decreased, the relative regression of tumors was also decreased. At 500 rads, no noticeable effect of radiation was found. This experiment was performed the earliest and after this observation, the radiation dose of 1,500 rads was fixed for all the other experiments. As mentioned earlier, this dose did not cause radiation sickness in any of these animals.

Discussion

Besides radiopotentiators and radiosensitizers, beneficial effects of hyperthermia in the treatment of cancer have been shown by many investigators.[8,14-19] Killing effects of hyperthermia on normal[8,20] and on neoplastic cells[21-23] *in vivo* and *in vitro* have been carefully studied. The relatively radioresistant S-phase cells are selectively radiosensitized by heat. Although hyperthermia kills both neoplastic and normal cells, rapidly cycling neoplastic cells tend to be more sensitive to hyperthermia than slowly turning normal cells. Unlike radiation, hyperthermia slso kills both hypoxic and well-oxygenated cells.[7,24] Nutrient deficient cells at low pH are more sensitive to heat.[25] These are likely to be hypoxic tumor cells, which may be out of cell cycle. This may constitute a basic rationale for the selective effect of heat on tumor cells.

Several possible mechanisms of hyperthermia cell killing have been proposed. Fajards et al[26] reported that hyperthermia produced focal cytoplasmic swelling, rupture of plasma membranes, and peripheral migration of heterochromatin. Westura and Dewey[22] indicated that protein molecules are particularly heat sensitive, and damage occurs primarily in chromosomes, at least in the cells engaged in DNA synthesis. Overgaard[27] proposed that the damage to the cell is caused by the activation of lysosomes which cause the release of their hydrolytic enzymes into cell cytoplasm. Other observations indicated that heat could increase membrane fluidity and membrane transport, and could decrease all membrane activity.[9,28,29]

Whatever the mechanism might be, in our studies heat showed an

Table I

EFFECTS OF HYPERTHERMIA, RADIATION AND MISONIDAZOLE ON THE HUMAN CARCINOMA OF CERVIX TRANSPLANTED IN ATHYMIC MICE

	Pre-treatment		Treatment Period		2 Weeks Post Treatment		4 Weeks Post Treatment		6 Weeks Post Treatment		8 Weeks Post Treatment		10 Weeks Post Treatment	
GROUP	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor
Untreated	4	1.3±0.1	4	1.5±0.2	4	1.8±0.3	4	2.0±0.3	4	3.1±1	4	4.1±1.1[a]		
Hyperthermia (A)	4	1.5±0.2	4	1.3±0.2	4	1.0±0.1	4	0.3±0.2	4	0.07[b]		–		–
(B)	3	2.0±0.5	3	2.0±0.2	3	2.2±0.3	3	2.5±0.3	3	3.0±0.5	3	3.2±0.3		
Radiation	12	1.1±0.3	12	1.1±0.3	11[c]	1.0±0.3	11	0.9±0.2	11	0.8±0.3	9[c]	0.4±0.6	9	.04±0.12
Hyperthermia and radiation	5	1.2±0.2	5	1.0±0.4	5	0.8±0.6	5	0.4±0.4	4[c]	0.25±0.1	4	0.1±0.1[b]		

a – due to large size of tumors mice were sacrificed.
b – only one mouse with tumor
c – mice found dead in the cage.

Table II

EFFECT OF HYPERTHERMIA AND THERAPEUTICAL AGENTS ON THE HUMAN SQUAMOUS CELL CARCINOMA OF THE CERVIX TRANSPLANTED IN ATHYMIC MICE

	Pre-treatment		Treatment Period		2 Weeks Post Treatment		4 Weeks Post Treatment		6 Weeks Post Treatment		8 Weeks Post Treatment	
GROUP	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor
Hyperthermia only	4	1.5±0.2	4	1.3±0.2	4	1.0±0.1	4	0.3±0.2	4	0.07[a]	–	–
Misonidazole alone	6	1.4±0.3	6	1.4±0.2	6	1.6±0.3	6	1.8±0.3	6	2.0±0.4	6	2.5±0.6
Misonidazole and hyperthermia	4	1.4±0.3	4	1.4±0.2	4	1.2±0.2	4	0.8±0.2	4	0.3±0.3	4	0.1[a]
Misonidazole and radiation	10	1.1±0.2	10	0.9±0.2	9[b]	0.3±0.1	9	0.04[a]		–		–
Indomethacin alone	4	1.0±0.1	4	1.2±0.1	3[b]	1.4±0.2	3	1.7±0.2	3	1.9±0.3	3	2.5±0.5
Indomethacin and hyperthermia	8	0.9±0.1	8	0.7±0.1	7[b]	0.8±0.1	7	0.6±0.03	7[c]			
Ibuprofen alone	4	1.1±0.1	4	1.3±0.1	4	1.4±0.2	4	1.6±0.3	4	2.1±0.3	4	2.8±04
Ibuprofen and heat	8	1.0±0.1	8	0.8±0.1	8	0.7±0.2	7[b]	0.6±0.2	7	0.3[a]		

a– only one mouse with tumor

b– one mouse found dead in cage

c– all mice were found dead–necroscopy showed no obvious cause.

Table III

EFFECT OF GRADED DOSES OF RADIATION COMBINED WITH ADRIAMYCIN ON HUMAN SQUAMOUS CELL CARCINOMA OF CERVIX TRANSPLANTED IN AHTYMIC MICE

	Pre-treatment		Treatment Period		2 Weeks Post Treatment		4 Weeks Post Treatment		6 Weeks Post Treatment		8 Weeks Post Treatment		10 Weeks Post Treatment	
Doses of Radiation	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor	Number of mice	Mean size of tumor
Untreated	5	1.3±0.1	5	1.5±0.3	5	2.0±0.2	5	2.8±0.3	5	3.5±0.8	5	4.3±0.8[a]		
500 rads	6	1.3±0.1	5[b]	1.4±0.2	5	1.2±0.1	5	1.6±0.3	5	2.1±0.7	5	3.4±0.8[a]		
750 rads	6	1.5±0.1	5[b]	1.5±0.1	5	1.5±0.1	5	2.0±0.1	5	2.4±0.2	5	3.5±0.6[a]		
1,000 rads	6	1.3±0.2	6	1.4±0.1	5	1.1±0.1	5[c]	0.6±0.2	5	0.3±0.3	5	0.3[d]		
1,500 rads	6	1.2±0.1	6	1.1±0.3	5[c]	0.5±0.5	5	0.1[d]±0.4	5	–		–		

a – due to large size of tumors mice were sacrificed
b – one mouse died due to anesthesia
c – one mouse was found dead in the cage
d – only one mouse with the tumor.

impressive effect on the tumor size. In one of our experiments where the tumors were large (2cm in diametre) at the beginning of treatment, hyperthermia failed to retard their growth. As the time in treatment was equal for all studies, it may be that the centre of the larger tumors did not reach 43°C during the treatment. Time and temperature relationship is a very important parameter when using heat treatment. Temperature of 43°C has been found to be most effective.[27,30] During our studies, the centre of the tumor measuring about 1cm in diametre reached 43°C within 15 minutes of treatment. These observations were the basis for our selection of treatment at 43°C temperature for 40 minutes.

Another important factor in heat treatment is thermo-tolerance. The cells can develop resistance to subsequent heat by prior heat treatment.[31,32] Once the cells become thermo-tolerant, it takes as much as three or four days to revert to their normal sensitivity or the decay of thermo-tolerance. It appeared that the decay of thermo-tolerance occurred faster in cells that were rapidly dividing than those that were not dividing.[33] To avoid thermo-tolerance, we treated tumors only twice a week. This also provided enough time for the decay of thermo-tolerance if any had developed.

The interaction between heat and irradiation is very complex and has been discussed at length by many investigators. Li et al[34] suggested that heat enhances the cell killing effect of radiation even after X-ray treatment. The mechanism of the radiosensitizing effect of post-irradiation heat treatment may involve the inhibition of potentially lethal damage repair. Hyperthermia inhibits the repair or radiation-induced single-strand breaks and radiation-induced chromosomal aberrations.[35-37] In other words, heat reduces the repair of sublethal damage and potentially lethal damage produced by radiation. Although Li et al[34] reported that heat after radiation inhibits the potential lethal damage, and heat treatment before radiation does not, our observations indicated no difference in the sequence. Heat treatment either before (within 30 minutes) or after (within 1 hour) reduced the size of the tumor equally. The maximum toxicity is observed when radiation is delivered simultaneously with heat, i.e., during the heat intervals.[38] Our method of combining the two treatments within a short period of time may be very close to the simultaneous treatment. The phenomenon of thermal tolerance during combined heat and radiation treatment is less important for low fractionated radiation doses (200-300 rads) than for high fractionated radiation doses.[30] In our studies, the most effective radiation dose was 1,500 rads, which was selected for all the experiments. Overgaard and Nielsen[39] observed that with 5 fractions, each consisting of radiation and heat, separated by 24 hours, the thermal enhancement ratio for a TCD50 was no greater than if the same heat dose had been given only once with a single radiation dose. However, when the fractionation interval was increased, supposedly allowing thermal tolerance decay, the effect of subsequent heat dose was greatly increased. Again, the spacing of treatment (twice a week) in our experiments might have avoided the thermal tolerance for heat radiosensitization, because the combination of hyperthermia with irradiation was found to be the most effective in regression of tumors in our investigation.

The cell killing potential of some drugs is enhanced subsequently by hyperthermia.[40-43] Hyperthermia is advantageous in targetting and localizing the principal effect of the drug, allowing greater tumor cell kill for a given systemic toxicity.

Misonidazole, a 2-nitroimidazole, an electron-affinic hypoxic cell sensitizer was found to be one of the best Flagyl compounds for the treatment of tumors.[44] It is postulated that its toxic effect is due to its reduction product induced by hydrated electrons in anoxia.[45] Ionized radiation produces a chemical reduction of misonidazole.[46] It minimizes the effect of oxygen as a reason for failure in the irradiation of hypoxic tumors. The optimal concentration of this drug is reached two hours after its administration.[47] Thus the radiosensitivity of the tumor is greatly increased when misonidazole is administered to animals before the radiation. In our experiments, the drug was given two hours before the radiation treatment, and the tumors from all the animals, except one tumor which had become very small, disappeared completely. In this study, misonidazole appeared to be an optimum sensitizer. When this drug was used in combination with hyperthermia, the regression of tumors was not as dramatic as when it was used with radiation. Furthermore, the results obtained by heat treatment alone were very similar to these results. It is our suggestion that the effect shown may be due to the heat alone. Obviously for this reason, we did not find any reference in literature where misonidazole was used in combination with hyperthermia.

Anti-inflammatory drugs such as indomethacin at high concentrations may inhibit phosphodiesterases[48] and allow accumulation of cyclic AMP with inhibition of cell division.[49,50] High concentrations of indomethacin may reach toxic levels and produce a non-specific inhibition of cells. Indomethacin given alone significantly slowed the tumor growth rate, but after the drug withdrawal, the tumor growth resumed. This phenomenon was also reported by Kantor and Hampton.[51] Indomethacin at its high concentrations was also not well tolerated by nude mice. Although the size of tumors regressed continuously when used in combination with hyperthermia, hyperthermia may further enhance its toxic effects, because the mice treated with this regimen died four weeks after the treatment.

Another non-steroid anti-inflammatory drug, Ibuprofen, which inhibits prostaglandin synthesis, reduces tumor growth and increases survival time, was used in this study.[52-54] In addition, the stimulation of cell swelling, cell aggregation, polymorphonuclear leukocyte locomotion, and lysosomal enzyme release in response to chemoattractant were inhibited by Ibuprofen.[55] Ibuprofen was found to be less tosic to mice, and when used in combination with hyperthermia gave better results than heat treatment alone or a combintion heat and indomethacin. All of the tumors, except in one mouse, regressed four weeks after the treatments. This drug when used alone did not show any significant effects on the growth rate of the tumors.

The precise mechanism of action of prostaglandins is still unclear. Exogenous prostaglandin E was found to inhibit replication of normal, transformed, and malignant cells *in vitro*[56-58] Santaro et al[59] demonstrated that 16, 16-dimethyl PGE_2, a long acting synthetic analogue of PGE_2, inhibited the growth of B-16 adenoma both *in vitro* and *in vivo*. They further demonstrated that systemic administration of dimethyl PGE_2 significantly inhibited tumor growth and prolonged the median survival in these mice. On the other hand, prostaglandins of E series have been shown to inhibit mitogen-induced stimulation, cytolysis, and antibody production by murine and himan lymphocytes,[60,61] suggesting that prostaglandins will subvert the immunological system. The results in our study were so inconsistent that no logical conclusion could be drawn.

In short, we have shown here some beneficial effects of hyperthermia in the treatment of human squamous cell carcinoma of the cervix transplanted in nude mice, especially when used in combination with radiation or a drug such as Ibuprofen. Although misonidazole produced dramatic results when used in combination with radiation, its results when combined with hyperthermia were not impressive. The results obtained by combination of indomethacin and hyperthermia were also similar to results obtained by using hyperthermia alone. We hope that this study may provide a broader selection for the treatment of this tumor in human patients.

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TEST OF GENIUS

Ask a friend to read this sentence slowly:

FINISHED FILES ARE THE RESULT OF YEARS OF SCIENTIFIC STUDY COMBINED WITH THE EXPERIENCE OF YEARS.

Then tell him to count aloud the F's in that sentence. Let him count them only once. How many?

A person of average intelligence finds three F's if you spotted four, you are above average. If you got five, you can turn up your nose at almost anybody. If you caught all six, you are properly a genius, and the question is whether you should spend your time taking tests like this ...