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Laboratory Investigation

Local delivery of minocycline and systemic BCNU have synergistic activity in the treatment of intracranial glioma

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Summary

Minocycline, a tetracycline derivative, has been shown to inhibit tumor angiogenesis through inhibitory effects on matrix metalloproteinases. Previous studies have shown this agent to be effective against a rodent brain tumor model when delivered intracranially and to potentiate the efficacy of standard chemotherapeutic agents. In the present study, the *in vivo* efficacy of intracranial minocycline delivered by a biodegradable controlled-release polymer against rat intracranial 9L gliosarcoma was investigated to determine whether it potentiates the effects of systemic 1,3-*bis* (2-chloroethyl)-1-nitrosourea (BCNU). Minocycline was incorporated into the biodegradable polymer polyanhydride poly[*bis*(*p*-carboxyphenoxy)propane-sebacic acid] (pCPP : SA) at a ratio of 50 : 50 by weight. The release kinetics of minocycline from the polymer were assessed. For the efficacy studies, female Fischer 344 rats were implanted with 9L glioma. Treatment with minocycline delivered by the pCPP : SA polymer at the time of tumor implantation resulted in 100% survival in contrast to untreated control animals that died within 21 days. Treatment with the minocycline-polymer 5 days after tumor implantation provided only modest increases in survival. The combination of intracranial minocycline and systemic BCNU extended median survival by 82% compared to BCNU alone ($p < 0.0001$) and 200% compared to no treatment ($p < 0.004$). We conclude that local intracranial delivery of minocycline from biodegradable controlled-release polymers inhibits tumor growth and may have clinical utility when combined with a chemotherapeutic agent.

Introduction

The treatment of malignant gliomas remains a difficult problem for clinicians, because these tumors are still among the most lethal neoplasms to treat. Conventional therapies, including surgery, radiotherapy, and chemotherapy, are not curative and yield only a modest impact on survival for most patients. Investigations aimed at elucidating molecular targets for therapeutics continue to be conducted, and among these, angiogenesis, the development of new capillary networks from the preexistent vasculature, has generated considerable interest [1–8]. Folkman and his colleagues in 1971 first postulated that tumors were dependent on angiogenesis for sustained growth, and consequently that inhibition of angiogenesis might reduce the development of tumors [9,10]. Subsequent studies demonstrated that angiogenesis is activated during multistage tumorigenesis before the emergence of solid tumors, and elaborate mechanisms exist for the maintenance of the neovascular network [11–14]. Malignant gliomas exhibit extensive microvascular proliferation and are among the most vascular of all solid tumors [2,15]. Therefore, we investigated the potential antineoplastic activity of minocycline, which has been demonstrated to inhibit angiogenesis.

Minocycline, a tetracycline derivative, was originally utilized clinically for its antibiotic effects. Previous studies have shown that minocycline also inhibited tumorigenesis, particularly through antiangiogenic mechanisms [16–21]. These antiangiogenic effects are exerted through the inhibition of matrix metalloproteinases, specifically collagenase and Type IV collagenase/gelatinase [22–24]. In addition

to its effects on angiogenesis, minocycline inhibits eukaryotic mitochondrial protein synthesis [25].

A previous study in our laboratory investigated the ability of minocycline to prolong survival in the rat 9L gliosarcoma brain tumor model [26]. This agent was delivered both systemically and by local intracranial delivery via the nonbiodegradable polymer, ethylene vinyl acetate (EVAc). The results of this study demonstrated that local intracranial delivery of minocycline was effective against 9L gliosarcoma when given simultaneously at the time of tumor implantation. Five days after tumor implantation, the combination of intracranial minocycline and either systemic 1,3-*bis* (2-chloroethyl)-1-nitrosourea (BCNU) or surgical resection resulted in prolongation of survival.

In the present study, we investigated the efficacy of local intracranial minocycline alone, delivered by a biodegradable polymer, poly[*bis*(*p*-carboxyphenoxy)propane-sebacic acid] (pCPP : SA), and in combination with systemic BCNU on survival of rats with intracranial 9L gliosarcoma. We found that survival was prolonged when rats were treated with minocycline-polymer at the time of tumor implantation, and when treated 5 days after tumor implantation with minocycline-polymer and a single injection of BCNU. In addition, we show in this model that tumor resection is not necessary to achieve prolonged survival when minocycline is locally delivered by this biodegradable polymer.

Materials and methods

Experimental design

A total of 68 female Fischer 344 rats, weighing 150–200 g (Charles River, Wilmington, MA) were utilized. The animals were anesthetized with an intraperitoneal injection of 3 ml/kg of a stock solution composed of ketamine hydrochloride (Abbott Laboratories, Chicago, IL) 25 mg/ml, xylazine (Phoenix Pharmaceutical, St. Joseph, MO) 2.5 mg/ml, and 14.25% ethyl alcohol in 0.9% NaCl. The 9L gliosarcoma was then placed in the brains of each rat as previously described [26]. Treatment was administered either at the time of tumor implantation or 5 days after tumor placement, when the tumor had established a substantial vascular supply, thereby mimicking the clinical setting more closely.

Three experimental protocols were used. For simultaneous implantation of minocycline-polymer and tumor, rats were divided into two groups: Group 1, treated ($n = 7$), received minocycline-polymer at the site of tumor implantation; Group 2, control ($n = 8$), received tumor implants only. Prior experiments had established that animals implanted with tumor and receiving empty polymers had the same outcome as did those implanted with tumor only. Therefore, the effects of empty polymers were not included in these experiments.

For treatment of established tumors, 5 days after tumor implantation rats were divided into two groups: Group 1, treated ($n = 15$), received minocycline-polymer implanted at the site of the established tumor; Group 2, control ($n = 8$), received no treatment.

For assessing the effect of minocycline-polymer and systemic administration of BCNU, 5 days after tumor implantation rats were divided into four groups: Group 1, minocycline-polymer ($n = 7$), received minocycline-polymer only; Group 2, combination treatment ($n = 7$), received minocycline-polymer and a single injection of BCNU; Group 3, systemic BCNU ($n = 8$), received a single injection of BCNU; and Group 4, control ($n = 8$), received no treatment.

The rats were kept in standard facilities with free access to food and water. After surgery, each rat was examined daily for behavioral changes and neurological deficits. Upon the death of each rat, brains were harvested and fixed in 10% formalin for at least 2 weeks. The area within the cerebral hemisphere containing the neoplasm was sectioned and stained with hematoxylin and eosin to confirm the presence of tumor.

Polymer preparation

pCPP : SA was used in combination with minocycline at a ratio of 50 : 50 by weight. pCPP : SA (Guilford Pharmaceuticals, Inc., Baltimore, MD) and minocycline (Sigma Chemical, St. Louis, MO), 500 mg each, were combined and dissolved in methylene chloride (Fisher Scientific, Pittsburgh, PA) at room temperature. This solution was dried in a vacuum desiccator for 1–2 h and stored at -20°C. The polymers were molded into disks measuring $3 \times 3 \times 1$ mm, weighing 10 mg each.

Release kinetics

Three pCPP : SA polymers, each containing 50% minocycline by weight, were used to characterize the release of minocycline. Each polymer was placed in

1.5 ml of Dulbecco's phosphate-buffered solution and incubated at 37°C. The buffer was replaced with fresh phosphate-buffered solution at 1–3, 5, 6, and 24 h, then daily for 2 weeks. The amount of minocycline released into the discarded buffer was measured immediately by spectrophotometric absorbance at a wavelength of 324 nm. A standard curve was constructed and used to determine the minocycline levels in the samples. The cumulative release was then calculated.

Intracranial tumor implantation and treatment

In the initial phase of each experiment, 9L gliosarcoma was excised from the flanks of carrier rats and cut into 1-mm^3 pieces. The female Fischer 344 rats were anesthetized, and their heads were shaved and prepared with povidone-iodine. A midline scalp incision was made and the coronal and sagittal sutures were identified. A burr hole 3 mm in diameter was drilled 3 mm lateral to the sagittal suture and 5 mm posterior to the coronal suture. The dura, cortex, and underlying white matter were resected with suction until the brainstem was visualized. A tumor piece was then placed into the defect. After hemostasis was achieved, the scalp incision was closed with surgical staples.

For the group receiving simultaneous implantation of tumor and minocycline-loaded polymer, the tumor piece was implanted first and the minocycline-loaded polymer was placed next to it.

The delayed treatment group received tumor alone on Day 0 and then underwent reoperation 5 days later for polymer implantation. On Day 5, the animals were anesthetized and prepared as described for the original implantation, the midline scalp incision was reopened and the burr hole was identified. A minocycline-loaded polymer was placed into the original defect, and the scalp incision was closed with staples.

Systemic drug administration

The BCNU (Bristol Meyers, NJ) solution was made according to the manufacturer's instructions. BCNU powder (100 mg) was dissolved in 3 ml of sterile anhydrous ethanol followed by the addition of 27 ml of sterile water. The final concentration of the BCNU solution was 3.3 mg/ml. In appropriate treatment groups, a single intraperitoneal injection at a dose of 10 mg/kg was administered 5 days after tumor implantation, either alone or with polymer treatment.

Statistical analysis

StatView v5.0 for Windows (SAS Institute, Inc., Cary, NC) was used to determine the differences between treatment groups. The Kruskal–Wallis test for survival analysis was used to determine significance.

Results

Release kinetics

The *in vitro* release kinetics of minocycline were studied over a 15-day time period (Figure 1). Twenty-six percent of the total drug load was released from pCPP : SA during this time frame, with 8% released at the end of Day 1. Minocycline was released from pCPP : SA at a rate of 1.7% of the total drug load per day, after the first 24 h.

Efficacy of simultaneous implantation of minocycline-pCPP : SA and 9L glioma

All control animals died within 21 days after tumor implantation, with a median survival of 14 days. The intracranial delivery of minocycline, via a 50%-loaded polymer, at the time of 9L gliosarcoma implantation resulted in a long-term survival of 100% (Figure 2). Histological analysis showed that all controls died of a large tumor mass, whereas no tumor was found in the treatment group when a representative sample was euthanized after 120 days.

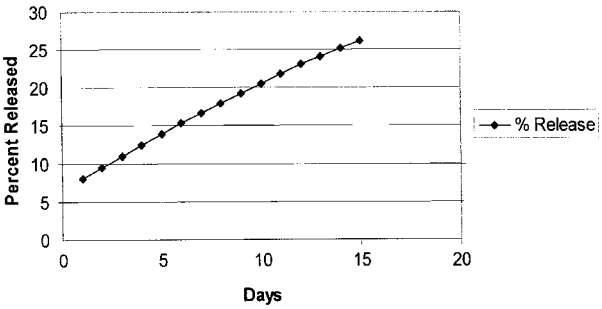

Figure 1. In vitro release kinetics of minocycline released from pCPP : SA (20 : 80) polymer discs (10 mg) loaded with 50% minocycline by weight. This experiment was conducted over a 15-day time period and each point represents the mean of three measurements.

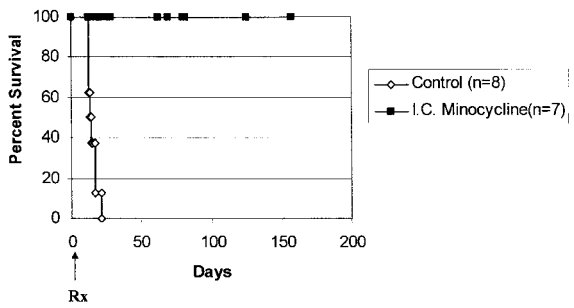

Figure 2. Kaplan–Meier survival curves for animals that received simultaneous tumor implantation and polymer treatment. On Day 0, seven female Fischer 344 rats received intracranial 9L gliosarcoma followed by an intratumoral implant of a 10-mg pCPP : SA disc loaded with 50% minocycline. The eight control animals received tumor only. I.C. = intracranial; Rx = time of treatment.

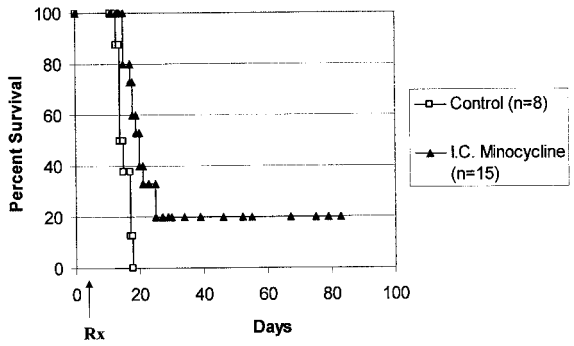

Figure 3. Kaplan–Meier survival curves for animals treated with polymer 5 days after tumor implantation. On Day 0, 15 female Fischer 344 rats received intracranial 9L gliosarcoma and on Day 5 they received an intratumoral implant of a 10-mg pCPP : SA disc loaded with 50% minocycline. The eight control animals received tumor only. Median survival time of the treatment group is significantly prolonged when compared with that of the control group ($p < 0.004$). I.C. = intracranial; Rx = time of treatment.

Efficacy of delayed minocycline treatment

When treatment was initiated 5 days after tumor implantation, survival in the minocycline group was minimally improved compared to the group receiving no treatment (Figure 3). All control animals died by Day 18, with a median survival of 14 days. The group treated on Day 5 with the minocycline-loaded polymer had a median survival of 20 days; 3 animals achieved long-term survival over 83 days. This modest increase in median survival was statistically significant ($p < 0.004$).

Efficacy of delayed minocycline and BCNU treatment

Systemic BCNU was used in combination with intracranial minocycline to determine whether the combination of the two agents would enhance their efficacy. As shown in Figure 4, the intracranial delivery of minocycline in combination with a single dose of systemic BCNU (10 mg/kg) resulted in a significant prolongation of the median survival to 42 days compared to 23 days in the group treated with systemic BCNU alone ($p < 0.0001$), 19 days in the group treated with intracranial minocycline alone ($p < 0.02$), and 14 days in the control group ($p < 0.004$). The dual treatment represented increases of 82%, 121%, and 200% in median survival time compared to systemic BCNU alone, intracranial minocycline alone, and no treatment, respectively. Systemic BCNU (10 mg/kg) alone increased median survival time by 64% compared to controls ($p < 0.0001$). Intracranial minocycline alone resulted in a modest increase of the median survival by 35% compared to controls ($p < 0.002$).

Postmortem analysis

Histological analysis of brain specimens confirmed the presence of tumor in animals at the time of death. Tumor histology did not differ between treatment groups and controls.

Discussion

Malignant gliomas of the central nervous system (CNS) continue to be a challenging clinical problem, in which the prognosis for patients remains dismal. The unique structure and physiologic and pharmacologic properties of the blood–brain barrier prevent the influx of molecules from the bloodstream into the brain, except for small, electrically neutral, lipid-soluble molecules. Since many chemotherapeutic agents are large charged molecules, tumoricidal drug concentrations usually cannot be delivered systemically without potential severe systemic side effects. The development of local CNS pharmacotherapy has provided a significant new treatment modality for CNS malignancies.

The use of controlled-release polymers to deliver pharmacotherapy interstitially to the CNS provides an alternative to systemic, intrathecal, or intraventricular administration. The advantages offered by controlled-release polymers include sustained high local tissue

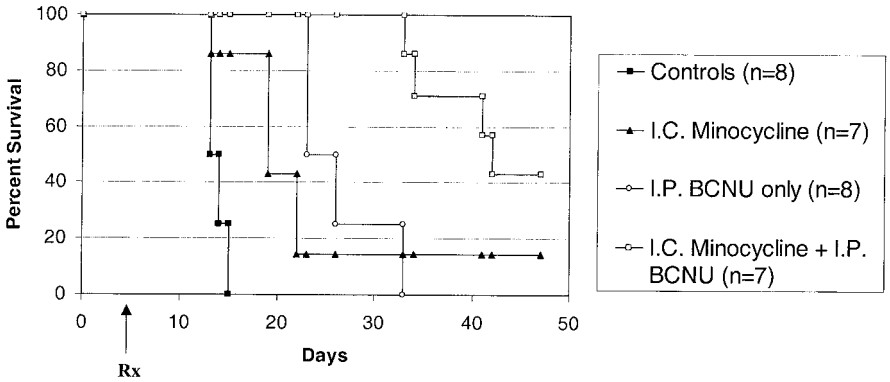

Figure 4. Kaplan–Meier survival curves for animals treated with either polymer alone or polymer in combination with systemic BCNU 5 days after tumor implantation. On Day 0, female Fischer 344 rats received intracranial 9L gliosarcoma and on Day 5 they received an intratumoral implant of a 10-mg pCPP : SA disc with 50% minocycline, with 50% minocycline and an I.P. injection of BCNU, or I.P. BCNU only. Survival of the 50% minocycline/I.P. BCNU group is significantly prolonged when compared with that of the control group ($p < 0.004$), 50% minocycline group ($p < 0.02$), and I.P. BCNU group ($p < 0.0001$). I.C. = intracranial; Rx = time of treatment; I.P. = intraperitoneal.

concentrations of drug without high systemic levels, bypass of the blood–brain barrier's tightly regulated environment, and reproducible kinetics [27–30]. *In vivo* studies have demonstrated the efficacy of local anti-tumor agents in experimental models [26,28,31]. Clinical trials in humans have also shown efficacy of chemotherapy delivered by biodegradable polymers [32,33].

Minocycline, a tetracycline derivative, has been utilized experimentally for its anti-tumor effects. Clinically, tetracycline derivatives have been used as antibiotics, since they inhibit bacterial protein synthesis by binding to the bacterial 30S ribosome. Several experimental *in vivo* cancer models, including the rat 9L gliosarcoma, rabbit VX2 carcinoma, rat Walker 256 sarcoma, and the Lewis lung carcinoma in mice, demonstrated the anti-tumor effects of tetracycline derivatives [16–18,26,34]. Previous studies have attributed the ability of minocycline to inhibit tumor growth to the inhibition of mitochondrial protein synthesis [25]. Additional studies have shown that minocycline exerts its effects through the inhibition of angiogenesis, including the inhibition of neovascularization in the rabbit cornea [16]. The anti-angiogenic effects of minocycline may be related to its ability to inhibit matrix metalloproteinases, and recent studies have shown that minocycline inhibits collagenase and Type IV collagenase/gelatinase [22–24]. Moreover, minocycline inhibits the growth of early-passage endothelial cells with minimal cytotoxic effects against tumor cells [17,22].

Since maligant gliomas are among the most vascular of solid tumors, a previous study in our laboratory was designed to determine whether the interstitial delivery of minocycline in combination with the nonbiodegradable polymer EVAc could prolong survival in a rat 9L gliosarcoma model. This study demonstrated that local interstitial treatment with a minocycline-EVAc polymer implanted at the time of tumor implantation extended median survival time compared to treatment with empty polymer, which was statistically significant. Treatment begun 5 days after tumor implantation had no effect on survival. After tumor was resected, however, median survival time was prolonged by 43% ($p < 0.002$). In addition, the combination of local intracranial minocycline-EVAc and systemic BCNU 5 days after tumor implantation prolonged the median survival by 93% ($p < 0.002$). Overall, this study demonstrated the efficacy of minocycline-EVAc when used in combination with chemotherapy and surgical resection of the tumor [26].

Based upon these findings, we investigated the role of minocycline in combination with a biodegradable polymer, pCPP : SA, against intracranial rat 9L gliosarcoma. Our data indicate that minocycline-pCPP : SA delivered at the time of tumor implantation resulted in long-term survival in 100% of rats. In contrast, polymer implantation 5 days after tumor implantation resulted in only a modest increase in the median survival time. These findings suggest that minocycline is effective alone when administered at the time of tumor implantation and before development

of a substantial vascular supply by the malignant glioma. Once the vascularization had occurred, however, minocycline was minimally effective. Synergistic activity was demonstrated when a combination of intracranial minocycline-pCPP : SA and systemic BCNU was administered 5 days after tumor implantation even without surgical resection unlike the previous study with minocycline-EVAc. The median survival time was extended by 200% ($p < 0.004$).

Unfortunately, malignant gliomas are usually not identified in the clinical setting until extensive vascular proliferation has occurred. This scenario was mimicked in our study by delaying treatment for 5 days to allow the 9L gliosarcoma to establish a vascular supply. Delayed treatment with intracranial minocycline-pCPP : SA alone is not effective against the tumor, suggesting that either the extensive vascular supply overwhelms the anti-angiogenic effects of minocycline, or a suboptimal dose of the drug is being delivered by the polymer. The cytoreductive effect of BCNU utilized concurrently with intracranial minocycline-pCPP : SA proved efficacious against the malignant glioma, suggesting that a critical reduction in the tumor mass is needed before minocycline can exert its therapeutic effect.

In summary, we describe the effective intracranial administration of minocycline from biodegradable controlled-release polymers. This tetracycline derivative, known to have anti-angiogenic effects, inhibits malignant glioma growth and prolongs survival of rats implanted with these neoplasms. When minocycline delivered by the controlled-release polymer is used in combination with a chemotherapeutic agent, the two agents act synergistically, and this dual treatment modality may have clinical utility.

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