Effects of Long-Term Oral Administration of Polymeric Microcapsules Containing Tyrosinase on Maintaining Decreased Systemic Tyrosine Levels in Rats

BINGLAN YU, THOMAS MING SWI CHANG

Artificial Cells and Organs Research Center, Faculty of Medicine, McGill University, 3655 Drummond Street, Rm 1006, Montreal, Quebec, Canada H3G 1Y6

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ABSTRACT: There is no effective treatment for melanoma, a fatal skin cancer occurring with increasing frequency. Dietary tyrosine restriction lowers systemic tyrosine and suppresses the growth of melanoma in mice, but this is not tolerated by human resulting in nausea, vomiting, and weight loss. We report here the successful use of oral polymeric microcapsules containing tyrosinase to lower the systemic tyrosine level in the rats. We found that microencapsulated tyrosinase incubated with intestinal content of rats selectively lowered the tyrosine level. We then studied the daily oral administration of microencapsulated tyrosinase in rats of one dose a day, two doses a day, and three doses a day over a period of up to 22 days. With three doses a day, the tyrosine levels in the test group decreased to 68.8% of the control group by day 4 and then decreased to 52.6% after this and remained at this level throughout the 22 days test period. This is the level shown earlier by other workers using dietary restriction of tyrosine to result in suppression of growth of melanoma. However, unlike dietary tyrosine restriction, oral tyrosinase microcapsules did not result in adverse effects nor significant differences in growth (weight gain) when compared to the control group. This approach can also be used for the lowering of systemic tyrosine in hypertyrosinemia, an inborn error of metabolism. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:831–837, 2004

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INTRODUCTION

There is at present no effective treatment for melanoma, a fatal skin cancer, now represents the fifth most common type of cancer in North America. The incidence rate of melanoma has risen dramatically in the last century in all countries, doubling every 10 years in many countries, and is now approximately 10 per 100,000 per annum in Europe, giving an approximate lifetime risk of 1 in 200.1 At present, there is no optimal treatment for this cancer. Adjuvant therapy tested includes immunotherapy, such as α-2b levamisole, vaccines, chemotherapy, autologous bone marrow transplantation, biochemotherapy, and chemoimmunotherapy.2–4 However, there is at present no conclusive method for the treatment of melanoma.

One unique characteristic of melanoma cells is that they need higher concentration of tyrosine for growth than that for normal cells.5 L-Tyrosine is an amino acid derived from protein degradation, dietary intake, and phenylalanine hydroxylation. Systemic tyrosine level can be reduced by the use of a low tyrosine diet.6 However, it takes a long time for this diet to lower systemic tyrosine. Furthermore, this diet is not well tolerated, resulting in nausea, vomiting, and weight loss. The injection of the enzyme, tyrosinase, which catalyzes the
conversion of L-tyrosine into L-dopa-quinone, by itself is also not practical because the short half-life of a few minutes after intravenous injection and thus requiring repeated injection resulting in immunological problems.

Similar problems related to diet restriction are also encountered in attempts to lower systemic tyrosine in hypertyrosinemia, an inborn error of metabolism. In the present study we analyze the possible use of oral administration of microencapsulated tyrosinase to avoid these problems. Artificial cells containing enzymes have been successfully used for experimental enzyme replacement in acatalasemia. However, this requires implantation of artificial cells. More recently, orally administered artificial cells containing phenylalanine ammonia lyase resulted in the control of phenylketonuria in an animal model. This is because of the interrupt of the enterorcirculation of phenylalanine. The present study is to see whether oral administration of artificial cells containing tyrosinase can be used to lower the systemic tyrosine level. This approach has potential advantages in enzyme therapy for the lowering of systemic tyrosine when compared with earlier approaches in experimental enzyme therapy, based on parenteral injections or extracorporeal circulation of blood. These include the absence of parental or surgical intervention, a large membrane surface area, and the improvement in enzyme stability. Artificial cells can be administered orally for substrate reduction in the gastrointestinal tract. Once microencapsulated within the artificial cells, the enzyme is protected from proteolytic enzymes in the intestinal tract. Our previous in vitro studies have shown that microencapsulated tyrosinase acts effectively in the conversion of tyrosine into L-dopa-quinone. The activity of microencapsulated tyrosinase is much higher than that of free tyrosinase solution at different pH and temperature. In the present report, these artificial cells are tested for their efficiency in decreasing tyrosine level in rat’s intestine contents at 37°C in vitro and also for the lowering of systemic tyrosine in rats after oral administration.

MATERIALS AND METHODS

Chemicals
Tyrosinase from mushroom (EC. 1.14.18.1, 5350 units/mg stated activity), hemoglobin from bovine (lyophilized powder) were purchased from Sigma Company (Oakville, Ontario, Canada). Collodion was purchased from Fisher Scientific Company (Whitby, Ontario, Canada). All other reagents were of analytical grade.

Preparation of Control Artificial Cells
Control artificial cells were prepared by the standard published method. Briefly, 1 g hemoglobin and 200 mg Tris were dissolved in 10 mL double-distilled deionized water and stirred with a metal rod until everything was dissolved. Gravity filtered the solution through a Waterman #42 filter into an Erlenmeyer flask; 2.5 mL of this 10 g/dL hemoglobin solution was taken, and was microencapsulated within spherical, ultrathin, cellulose nitrate membrane. Without tyrosinase, loaded microcapsules were administered orally to the control group. All control artificial cells were prepared daily and stored in 1% v/v Tween 20 solution at 4°C until use.

Preparation of Tyrosinase-Loaded Microencapsules for Oral Administration
1.907 mg of 5350 units/mg tyrosinase was dissolved in 5 mL 10% hemoglobin solution, and then followed the methods described above to immobilize tyrosinase in collodion membrane microcapsules. Microcapsules prepared as a 50% suspension for later feeding. Tyrosinase-loaded microencapsules were administered orally to test group. Before oral administration for both control group and test group, artificial cells suspended in 1% of Tween 20 were washed and resuspended in 0.1 M Tris·HCl buffer (pH 8.5). The total volume of artificial cells for feeding was 1 mL containing tyrosinase 1020 U/mL. This was suspended in 1.5 mL Tris·HCl buffer. This buffer was enough to protect the microencapsulated tyrosinase during its passage through the stomach with its acidic medium. For the in vitro studies, three types of tyrosinase microcapsules were prepared with tyrosinase concentration of 450, 670, and 900 U/mL.

In Vitro Studies Using Rat Intestinal Juice
Fasted male Sprague-Dawley rats (245–260 g) purchased from Charles River (St. Constant, Quebec, Canada) were anesthetized with intraperitoneal injection of pentobarbital (Somnotol, 65 mg/kg). The whole length of the small intestine was removed intact with a surgical scissor. The
contents were gently expressed into polypropylene centrifuge tubes stored on ice. These tubes were centrifuged at 1500 g for 10 min at 4°C, and supernatant was recovered for the following experiment. Microencapsulated tyrosinase at different concentration of 450, 670, and 900 U/mL was incubated with rat’s intestine juice at 37°C, and then samples were taken at different time intervals. Then, 10% trichloroacetic acid (TCA) was added to stop the reaction. After centrifuging the sample, the tyrosine level in the supernatant was measured by fluorometric method using Perkin-Elmer Luminescence Spectrometer LS50B.17,18

Animal Studies

Fasted male Sprague-Dawley rats (130–150 g) were used in this study. They were kept in a controlled 12-h light/dark environment with food and water ad libitum. All rats were acclimatized for at least 3 days prior to use. All animal experiments were performed according to the regulations of McGill University on Animal Care. Two groups (five rats in each group) were studied: (1) control group: each rat received oral administration of artificial cells containing no enzyme; (2) test group: each rat received oral administration of artificial cells loaded with tyrosinase.

In the following experiments, the blood sample was taken at 4:00 p.m. on day 0. No artificial cells were administrated on that day. From that day on, for daily dosing, artificial cells were given at 10:00 a.m. using stomach feeding tubes. Blood samples were taken 6 h after oral administration of the artificial cells every 2 days. The procedures for blood collection from lateral saphenous vein were as follows. First, the animal was placed in a restrainer. Then, the hind leg was extended and the limb was fixed by holding fold of skin between tail and thigh. The hair on outer surface of lower hind leg was shaved to visualize the vein. Vaseline was applied on skin prior to nick to prevent wicking of blood onto skin and to ease collection. The vein was nicked perpendicularly with a 25-gauge needle and the blood sample (about 1 mL) was collected by microhematocrit (capillary) tube. The plasma in each blood sample was separated from the blood and placed in a 1.5 mL plastic tube, then stored at -80°C until analyzed for tyrosine concentration.

For twice daily dosing, artificial cells were given at 10:00 a.m. and 4:00 p.m., and blood samples were taken every week (days 7, 14, and 21) just before the second feeding for 21 days. For three times daily dosing, artificial cells were administrated at 10:00 a.m., 2:00 p.m., and 6:00 p.m. for 22 days. Blood samples were taken on days 4, 8, 11, 15, 18, and 22 just after the second feeding.

Statistical Analysis

Data were expressed as mean ± SD. The differences of tyrosine concentration in rat’s plasma between the control group and the test group at the same time point were determined by using Student’s t-test within ANOVA and Bonferroni correction, and considered significant at p < 0.05.19

RESULTS

Incubation with Rat’s Intestine Juice In Vitro

This is to analyze the ability of the microencapsulated tyrosinase to lower tyrosine level in intestinal juice before using this for oral administration. We took fresh intestine juice from anesthetized rat, and incubated this with artificial cells at 37°C in a shaker.

Our results (Fig. 1A) showed that in both groups there was an initial decrease in tyrosine level due to the dilution of the intestine juice by the microcapsule suspension. After this, in the case of the control group the tyrosine level increased from 177.58 ± 29.92 mg/dl at the beginning to 219.76 ± 15.21 mg/dl at 33 min. The intestinal juice contains high concentrations of proteins, enzymes, polypeptides, and peptides.11 Intestinal trypic enzymes break these down into amino acids including tyrosine, thus the increase in tyrosine level.11 For the test group, tyrosine concentration in the intestinal fluid fell from the original concentration of 200.25 ± 10.16 mg/dl to 73.34 ± 14.72 mg/dl at 33 min. When we increased the amount of microencapsulated tyrosinase (Fig. 1B,C), tyrosine concentration in the test group can be maintained at the low level during the length of the experiment. Thus, in Figure 1B, for the control group, tyrosine level in the intestinal juice was 103.07 ± 6.83 mg/dL at the beginning and increased to 201.62 ± 11.03 mg/dL at 33 min. For the test group the tyrosine level fell from 118.98 ± 16.47 mg/dL to 56.55 ± 8.21 mg/dL. Figure 1C showed that in the control group, the tyrosine level increased to 1.5 times the original level. In the test group, because of the even larger amounts of tyrosinase microcapsules, the tyrosine
level decreased to even lower level when compared to that in Figure 1B. These results allow us to establish the minimal dosage needed for the next study where we administer the tyrosinase microcapsules orally to rats. Thus, we used an oral dosage of 1020 U/mL that is slightly higher than the highest dosage of 900 U/mL in the in vitro study to compensate for less efficient mixing in the intestine.

Animal Studies in Oral Feeding with Different Dosages

There was no significant change with daily dosing in tyrosine concentration in both groups during the 10-day study (Fig. 2A). The tyrosine concentration in plasma, as all other amino acids, tends to fluctuate even though the time of feeding and plasma collection are the same. This fluctuation is a normal phenomenon in all rat studies. Thus, we take tyrosine level in the control group as 100%, the other data are expressed as percentage of the original activity. Each group was given either control microcapsules containing no tyrosinase (control group) or microencapsulated tyrosinase (test group) daily for 10 days. Both groups of rats were fed on regular rat chow. Figure 2A showed plasma tyrosine concentration (%) taken 6 h after the daily oral administration over the 10 days period. Statistical analysis showed that there were no significant differences between the two groups. Thus, one dose a day was not effective in lowering the plasma tyrosine levels. The body weight gain of normal and those administered artificial cells orally once a day did not change significantly when compared to the control group over the 10 days.

Next, we studied the use of twice daily dosing for rats fed on regular rat chow. In this study, there was significant decrease in tyrosine level in the test group starting from the first week (Fig. 2B). Plotting the results as percent of control group, at day 7 the tyrosine concentration in the test group was decreased to 85.7% of that in the control group. At day 14 and day 21, tyrosine concentration was further decreased in the test group to 62.9 and 55.8%, respectively. Our results showed that microencapsulated tyrosinase was effective in lowering systemic tyrosine level. The gradual decrease of the systemic tyrosine level is because of the time taken for the large intracellular pool of tyrosine to equilibrate with the extracellular pool. After 1 week of oral administration, there was significant decrease ($p < 0.005$) in tyrosine level in the test group compared to the control group. By continuing this treatment until day 21, there was further increase in significance ($p < 0.0005$). There was no significant difference in the rate of growth as shown by weight gain during the 21-day experiment period between the control and test group. Unlike weight loss using tyrosine restricted diet,20 we found that the rats given oral tyrosinase artificial cells grew at the same rate as the control group.
To optimize and increase the rate of removal of systemic tyrosine, we increased to three times daily dosing. Figure 2C showed that there was much faster decrease in plasma tyrosine level in the test group when compared to that given two doses daily. Thus, on day 4, tyrosine levels in the test group decreased to 68.8%. By day 18 and 22, the systemic tyrosine level decreased to 56.8 and 52.6%, respectively. Our results showed that three times daily dosing can markedly lower tyrosine concentration in rat’s plasma from day 4 to a level that would inhibit the growth of melanoma. At day 4, there was already a significant decrease \((p < 0.05)\) in the test group when compared to the control group. The test group did not lose any weight. Instead, they gained weight with a weight gain curve identical to that of control group (Fig. 3). No abnormal effect or behavior was observed in both groups. This again showed that even at this increased dosage, there were no gross adverse effects when compared to the control.

**DISCUSSION**

Previous studies by other groups indicate that tyrosine and phenylalanine restrictive diets decrease tumor growth and metastasis and increase the survival of B16 melanoma-bearing mice.\(^{21-23}\) One of the main problems with these low amino acids diet is that it is difficult to sustain in humans. These restrictive diets cause nausea, vomiting and malnutrition, and weight loss in the severely ill patients. Other antitumor agents specific for malignant melanoma has been studied by many research groups. Inoue et al.\(^ {24}\) showed that 4-S-Cysteaminylphenol (4-S-CAP) is one of the most promising antimelanoma agents. Tyrosinase converts 4-S-CAP into a toxic metabolite with melanocytotoxicity and thus inhibits the growth of B16 melanoma. However, the hypotensive effect of 4-S-CAP limits the dose that can be administered \textit{in vivo}. Jordan et al. use a prodrug, nitrogen mustard, to incorporate into tyrosinase substrates for the treatment of malignant melanoma, but still have some limitations in drug selective liberation.\(^ {25}\) Castelli et al. use melanoma-associated antigens (MAA) recognized by T cells for boosting natural immune recognition of tumor cells.\(^ {26}\) The therapeutic potential of MAA still needs to be fully exploited to reach an effective and long-lasting \textit{in vivo} immune control of melanoma growth and progression.
In our study, polymeric membrane artificial cells to microencapsulate tyrosinase are used to lower tyrosine level in the body system. Oral uses of polymeric membrane artificial cells containing other enzymes have already been demonstrated in this laboratory and offer several advantages over other methods. Because polymeric membrane artificial cells pass through the intestine and are excreted once they have carried out their functions, it is easier to ensure their safety in patients. Furthermore, earlier clinical results of oral administration of other enzyme microcapsules here have already shown their safety. Our present studies have shown that microencapsulated tyrosinase can effectively lower tyrosine level in rat’s intestine contents in vitro. We use this in vitro result to establish an approximate dose for use in the animal studies. In vivo studies, the results for daily dosing, twice daily dosing, and three times daily dosing are compared. Based on the new theory of enterorecirculation of amino acids, the major source of intestinal amino acids could be from gastric, pancreatic, intestinal, and other intestinal secretions. Tryptic digestion converts the large amount of proteins, enzymes, and polypeptides in the secretion into amino acids. These amino acids are then reabsorbed back into the body as they pass down the intestine. This forms a large enterorecirculation of amino acids between the body and intestine. Therefore, in our experiments artificial cells loaded with tyrosinase are used to remove tyrosine from the amino acid pool in the intestinal tract and prevent its reabsorb into the body amino acids pool, thereby lowering systemic tyrosine level. Our results have shown that two or three times daily dosing can effectively reduce the systemic blood tyrosine level and keep it at that low level as long as the oral administration is continued. This experimental result also shows promise for the lowering of high systemic tyrosine level in hypertyrosinemia, an inborn error of metabolism.

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