The Effects of RAD001 on Neuroendocrine Differentiation and Prostate Cancer Progression

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ABSTRACT

Prostate cancer is the second leading cancer in men in the United States. Currently, the primary mode of treatment for advanced prostate cancer is hormone therapy (androgen ablation or inhibition of androgen action). For the majority of men, hormonal therapy induces an immediate remission of the cancer, within three years of treatment, an androgen-independent or castrate-resistant form of prostate cancer typically emerges. Recently, studies have suggested that neuroendocrine differentiation may contribute to the development of androgen-independent prostate cancer. This pathway has been shown to be activated by androgen deprivation in LNCaP cells, and additionally, rapamycin derivative RAD001 has been shown to inhibit the pathways of neuroendocrine differentiation and progression. Based upon this observation, we hypothesized that androgen deprivation induces neuroendocrine differentiation, which contributes to androgen-independent prostate cancer growth; additionally, inhibition of the molecule mTOR by rapamycin may inhibit neuroendocrine differentiation and, therefore, androgen-independent prostate cancer progression.

INTRODUCTION

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MATERIALS AND METHODS

1. Cell culture

LNCaP cells (American Type Culture Collection, Manassas, Virginia) were cultured in RPMI (16870; Invitrogen Corp., Carlsbad, California) supplemented with 10% fetal bovine serum (31086; Atlanta Biologicals, Lawrenceville, Georgia), 1% sodium pyruvate (11560-070; Invitrogen Corp., Carlsbad, California), and 1% penicillin-streptomycin (15140 Invitrogen Corp., Carlsbad, California). The cells were cultured at 37°C in humidified atmosphere with 5% CO2 and were passaged when flasks reached approximately 95% confluency.

2. Infection of LNCaP cells into animals

Twenty 8-week-old athymic nude mice were obtained from Maryland (NCI-Frederick, Frederick, Maryland) and housed in the vivarium under normal conditions. Mice had free access to a normal diet and water supply. The experiment was approved by the University Committee on Animal Resources.

Mice were injected subcutaneously with LNCaP cells prepared in the following manner: when flasks of cells were 100% confluent, adherent cells were detached with 1x trypsin (25200, Invitrogen Corp., Carlsbad, California), re-suspended in RPMI and counted using a cell counter Vi-Cell XR. The concentration of cells was found to be 5.22 x 10^6 viable cells/mL. For each injection, 192 µL of cell suspension was mixed in 100 µL Matrigel (354234, BD Biosciences, San Jose, California) and loaded into a syringe. The Matrigel/cell mixtures were kept on ice until the injections were administered. The final concentration of LNCaP cells was 1.0 x 10^6 cells per injection. Each mouse was given one injection in each flank region.

Concurrent with the injections, testosterone supplement was implanted subcutaneously in the inguinal region to facilitate hormone-dependent tumor growth. Testostosterone (T-1500, Sigma-Aldrich Corp., St. Louis, Missouri) was prepared in Silastic® laboratory tubing (Dow Corning, Midland, Michigan) and implanted.

3. Castration

Nine weeks after injections of LNCaP cells, sixteen mice had developed visible tumors ranging in size from 5 mm to 22 mm. These mice were castrated, and their testosterone implants were removed.

4. Administration of experimental substance

Following castration, the mice were divided into two groups, each containing eight mice. Each experimental group was administered the experimental substance while the control group received a placebo. Administration of the experimental substance, RAD001, and the placebo began immediately after castration. Substances were administered briefly by oral gavage. The experimental group received RAD001 while the control group received only vehicle. RAD was obtained from Novartis formulated as a microemulsion preconcentration at 20 mg RAD per 0.25g microemulsion preconcentration. Both RAD and the placebo were administered at a concentration of 10 µg with the total administration volume dependent on the mass of the mouse. Administration of substance continued until the animal died naturally but ceased by 20 weeks after castration. At 20 weeks after castration, all surviving mice were sacrificed.

5. Analysis of tissue

Tumor tissue was collected after the mice were sacrificed. The tissue was fixed in formalin and embedded in paraffin blocks. An H & E stain and a DAB stain for the neuroendocrine marker Chromogranin A were performed on each section.

RESULTS AND DISCUSSION

Typically, when implanted into a preclinical model, hormone sensitive tumor growth occurs until it is deprived of androgens at which point tumors begin to regress. Following regression, however, many tumors once again increase in size. It is hypothesized that at a particular point following castration, the androgen-independent tumor cells begin to predominate and cease the tumor to grow in size for some period of time. If RAD001 properly inhibits castrate-resistant tumor growth in this model, then we would expect xenografts in treated animals to not experience androgen-independent growth.

Our results showed no significant difference between the tumor growth curves of the experimental and control groups. (See Graph 1.) This outcome suggested that tumor growth following castration was not inhibited in the experimental group more than in the control group. A statistical analysis of tumor growth was performed using a two-sample T-test to select time points during the experiment. Results from this analysis are shown in Table 1. A P value < 0.05 implies that the two sample populations differ significantly. If this experiment were to verify our hypothesis, the experimental and control groups ought to differ significantly in tumor size during the later time points following castration. However, the smallest P value obtained using the T-test was 0.15, suggesting that tumor sizes of the two groups were

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<th>Day 78</th>
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Table 1. Mean values and standard error of tumor measurements were used in a two-tailed two sample T-test. T-test table shows the results.

P<0.05 indicates a significant difference. P>0.05 indicates no significant difference between the values. The T-test was performed at various time points starting the day of castration when treatment began (Day 0).

Graph 1. Tumor growth curve in both experimental (RAD) and control (RAD) mice. Tumors were measured biweekly beginning when the tumors were palpable. Mice were castrated around Day 70 after injection of LNCaP cells. No significant difference was seen between the Rad and the control group.
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ABSTRACT

 Approximately 242,000 men in America will be diagnosed with prostate cancer in 2009. Androgen ablation (deprivation) is currently the primary method of treatment for advanced prostate cancer. Though in most cases therapy is initially effective, the cancer frequently recurs in an androgen-independent state. Previous studies have suggested that neuroendocrine differentiation contributes to androgen-independent prostate cancer development. Notably, others have documented a potential role for the PI3 Kinase/AKT/mTOR pathway in neuroendocrine differentiation. In this study, we sought to explore the cancer, however, by investigating this pathway with a novel inhibitor RAD001, a drug shown to have inhibitory effects on the pathway. We used a mouse model of prostate cancer to determine whether inhibiting a crucial signaling pathway by RAD001 (a derivative of rapamycin which blocks the function of the molecule mTOR) impedes neuroendocrine differentiation and androgen-independent prostate cancer development. Our study demonstrated no significant impact of RAD001 on neuroendocrine differentiation and prostate cancer progression because we were unable to detect a significant difference between the group treated with RAD001 and the control group treated with a placebo. Direction for future studies is discussed.

INTRODUCTION

Prostate cancer is the second leading cancer in men in the United States. Currently, the primary mode of treatment for advanced prostate cancer is hormone therapy (androgen ablation or inhibition of androgen action). For the majority of men, hormonal therapy induces an immediate remission of the cancer, however, within three years of treatment, an androgen-independent or castrate-resistant form of prostate cancer typically emerges. Recently, studies have suggested that neuroendocrine differentiation may contribute to the development of androgen-independent prostate cancer. This pathway has been shown to be activated by androgen deprivation in LNCaP cells, Additionally, rapamycin derivative RAD001 has been shown to inhibit the pathways in prostate cancer. Based upon this observation, we hypothesized that androgen deprivation induces neuroendocrine differentiation, which contributes to androgen-independent prostate cancer growth; additionally, inhibition of the molecule mTOR by rapamycin may inhibit neuroendocrine differentiation and, therefore, androgen-independent prostate cancer progression. This hypothesis was tested in vitro using a mouse model of prostate cancer. Mice were injected subcutaneously with LNCaP cells, an androgen-dependent human prostate cancer cell line, which induce tumor development. The mice were castrated after tumor development. Animals in the experimental group were administered RAD001, a derivative of rapamycin, to determine whether androgen-independent tumor recurrence was inhibited. Tissue was collected as the mice died. Twenty weeks after castration, all surviving mice were sacrificed, and tumor tissue was examined for presence of neuroendocrine markers. An absence of neuroendocrine markers in the experimental group receiving RAD001, and conversely an absence of neuroendocrine cells in the control group, supported the hypothesis that inhibition of the PI3 Kinase/AKT/mTOR pathway inhibits neuroendocrine differentiation, which prevents androgen-independent prostate cancer development.

MATERIALS AND METHODS

1. Cell culture LNCaP cells (American Type Culture Collection, Manassas, Virginia) were cultured in RPMI (16180; Invitrogen Corp., Carlsbad, California) supplemented with 10% fetal bovine serum (S1068, Atlanta Biologicals, Lawrenceville, Georgia), 1% sodium pyruvate (11360-070; Invitrogen Corp., Carlsbad, California), and 1% penicillin-streptomycin (15140, Invitrogen Corp, Carlsbad, California). The cells were cultured at 37°C in humidified atmosphere with 5% CO2 and were passaged when flasks reached approximately 95% confluency.

2. Injection of LNCaP cells into animals Twenty-eight-week-old athymic nude mice were obtained from Maryland (NCI-Frederick, Frederick, Maryland) and housed in the vivarium under normal conditions. Mice had free access to a normal diet and water supply. The experiment was approved by the University Committee on Animal Resources.

Mice were injected subcutaneously with LNCaP cells prepared in the following manner: when flasks of cells were 100% confluent, adherent cells were detached with 1x trypsin (25280, Invitrogen Corp., Carlsbad, California), re-suspended in RPMI and counted using a cell counter Vi-Cell XR. The concentration of cells was found to be 5.2 x 108 viable cells/ml. For each injection, 192 µl of cell suspension was mixed in 100 µl Matrigel (354236, BD Biosciences, San Jose, California) and loaded into a syringe. The Matrigel/cell mixtures were kept on ice until the injections were administered. The final concentration of LNCaP cells was 1.0 x 107 cells per injection. Each mouse was given one injection in each flank region. Concurrent with the injections, testosterone supplement was implanted subcutaneously in the interscapular region to facilitate hormone-dependent tumor growth. Testosterone (T-1500, Sigma-Aldrich Corp., St. Louis, Missouri) was prepared in Silastic® laboratory tubing (Dow Corning, Midland, Michigan) and implanted.

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Tumor tissue was collected after the mice were sacrificed. The tissue was fixed in formalin and embedded in paraffin blocks.

RESULTS AND DISCUSSION

Typically, when implanted into a preclinical model, hormone sensitive tumor growth stops until it is deprived of androgens at which point tumors begin to regress. Following regression, however, many tumors once again increase in size. It is hypothesized that at a particular point following castration, the androgen-independent tumor cells begin to predominate and cause the tumor to grow in size for the following androgen deprivation.

If RAD001 properly inhibits castrate-resistant tumor growth in this model, then we would expect xenografts in treated animals to not experience androgen-independent growth.

Our results showed no significant difference between the tumor growth curves of the experimental and control groups (See Graph 1). This outcome suggested that tumor growth following castration was not inhibited in the experimental group more than in the control group. A statistical analysis of tumor growth was performed using a two-sample T test at select time points during the experiment. Results from this analysis are shown in Table 1. A P value < 0.05 implies that the two sample populations differ significantly. If this experiment were to verify our hypothesis, the experimental and control groups ought to differ significantly in tumor size during the later time points following castration. However, the smallest P value obtained using the T-test was 0.15, suggesting that tumor sizes of the two groups were similar.
insignificantly different at all points during the experiment. Because the sample size for both populations was small (n=16), a T-Test, which was meant to analyse larger populations, may not be the most accurate means of analysis. Therefore, a Wilcoxon Rank Sum Test was performed on the data because it is meant for small population sizes. In the Wilcoxon test, P=0.05 was considered a significant difference. However, the smallest P value obtained using the T-test was 0.16, suggesting that tumor sizes of the two groups were insignificantly different at all points during the experiment.

Figure 1 shows representative sections of RAD001-treated and placebo-treated tumor tissue stained with hematoxylin and eosin (H&E). As shown, no difference was detected between the two groups. Figure 2 shows tissue sections stained for the same neuroendocrine marker Chromogranin A. Section A is from a mouse in the experimental group and B is from a mouse in the control group. No CGA positive cells were found in sections from the experimental group, and a total of only three CGA positive cells were found in sections from the control group. An absence of CGA positive neuroendocrine cells initially appears to support our hypothesis. (Recall that we are using RAD001 to interrupt the PI3K/AKT/mTOR pathway which is crucial to neuroendocrine differentiation. If the pathway is interrupted, neuroendocrine cells should not be found in the tumor tissue). However, we would expect that neuroendocrine cells would be consistently present and at higher proportions in the control group than was observed. Therefore, we do not have sufficient evidence to conclude that in this experiment, RAD001 successfully inhibited neuroendocrine differentiation.

The xenograft model of human LNCaP prostate cancer cells in a mouse is well-established.11 Additionally, LNCaP cells, which contain androgen-dependent, are known to exhibit neuroendocrine phenotype when deprived of androgens for extended periods of time. Therefore, we must consider why this experiment yielded unexpected results.

LNCaP cells have been shown to exhibit neuroendocrine characteristics when cultured in an androgen-deprived state for prolonged periods of time. However, evidence for the same phenomenon in xenograft models using LNCaP cells is scant and conflicting.12-14 Several studies have reported that LNCaP xenograft tumors in castrated male mice contain significantly more neuroendocrine cells than tumors in non-castrated male mice. Other studies have been unable to corroborate this result; instead, a decrease in the number of androgen-independent xenograft tumors using LNCaP cells. In future experiments, androgen-dependent cell lines other than LNCaP could be used for xenograft creation with the expectation that the neuroendocrine phenotype will be induced following androgen deprivation. It was expected that the tumor sizes would decrease following castration and in the control group, would begin to grow again in an androgen-independent state following regression. This effect, which is a well-documented outcome of androgen-deprived prostate cancer progression, was paradoxically not observed in this study. That is to say, our control arm did not behave in the way we expected. It is possible that the castration was inadequate; though unlikely, there is a possibility that the orchectomy ineffectively removed the testes, or that the tubing in the equipment was clogged and the aperture was impaired prior to removal. This would result in incomplete testosterone supplement removal. Therefore, suboptimal androgen deprivation would result in the continued growth of androgen-dependent growth of the tumor cells, and the mice would not actually have been androgen deprived. Serum testosterone levels could have been analyzed to ensure androgen deprivation. It is also possible that the mice were exposed to exogenous androgens in their environment, perhaps through their diet. A more likely scenario would be a cell line contaminant. It is possible that even if an androgen-independent cell line contaminated the LNCaP population, and the resulting tumor did not consist of androgen-dependent cells as we intended. This could be tested by examining the cells for characteristic LNCaP markers, such as the androgen receptor (AR) which would be present on LNCaP cells, but not androgen-independent lines such as PC3 and DU145 cells.

A complicating factor in the analysis of our results was that most of the mice (13 out of 16) died before the projected endpoint was reached. While this is unfortunate, it is not uncommon. The mouse used for treatment with RAD001 did not survive for half of the anticipated experiment timeline. A possibility is that the mice simply did not live long enough for the androgen-dependent tumor to regress and the androgen-independent tumor to develop. Had they survived to the endpoint (20 weeks after castration), perhaps we would have observed not only the characteristic growth curve discussed above, but also the expected proportion of neuroendocrine cells in the control group. It should be noted that the excessive tumor size is ultimately what caused these mice’s demise. Had the tumors been smaller, the animals likely would have survived longer. So, death of the mice is secondary to the lack of tumor regression observed. Furthermore, the initial inoculant’s size injected into the mice could have been in excess 1x10^6 cells were injected into each flank. Perhaps using such a large number of cells caused the tumors to grow too large. We observed that the bulky tumors became necrotic, and many mice died from the burden of such tumors. If the inoculant’s size were smaller, perhaps the cells in the tumor would not necrotic and could be more easily manipulated by the experimental drug. Additionally, we were unable to detect a significant number of neuroendocrine cells in the control group, so it is not possible to determine whether the RAD001 was effective in the experimental group. We have shown that androgen-deprived LNCaP cells exhibit a neuroendocrine phenotype in vitro; however, there is conflicting data concerning whether LNCaP tumors actually exhibit neuroendocrine cell phenotype in vivo. Perhaps the in vivo conditions were not appropriate for the expression of neuroendocrine cells. To more definitively answer this question, we could examine more sections of tissue through the entire section. In further studies, we could use different methods for detecting neuroendocrine cells, such as immunohistochemistry for other neuroendocrine markers, like NE, or perform RT-PCR on non-formalin fixed tissue.

In essence, the results garnered in this study were inconclusive; the control group, which did not receive RAD001 treatment, did not behave in the expected manner. Therefore, there were no meaningful comparisons which could be drawn between the control group and the experimental group to determine whether the experimental drug did indeed have an effect on neuroendocrine cell differentiation as was originally hypothesized. It is unclear at this point why the control group behaved unexpectedly; as discussed, problems could have arisen with the original LNCaP cells that were engrafted into the recipient mice, or a complication could have arisen because of the overgrowth tumor observed in some of the animals. In future studies, the cell line ought to be evaluated prior to inject to verify that they are the appropriate phenotype. Additionally, primary cells derived from a patient could be used to avoid changes that occasionally occur in immortalized cell lines.

In subsequent studies, we could examine, at the molecular level, whether the PI3K/AKT/mTOR pathway was active in the control and whether it was, indeed, inhibited in the mice treated with RAD001. This analysis could be done by evaluating activity of the molecule mTOR and other downstream mTOR substrates.15 We could also determine the actual concentration
insignificantly different at all points during the experiment. Because the sample size for both populations was small (n=16), a T-Test, which was meant to analyse larger populations, may be the most accurate means of analysis. Therefore, a Wilcoxon Rank Sum Test was performed on the data because it is meant for small population sizes. In the Wilcoxon test, P=0.05 was considered a significant difference. However, the smallest P value obtained using the T-test was 0.16, suggesting that tumor sizes of the two groups were insignificantly different at all points during the experiment.

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A complicating factor in the analysis of our results was that most of the mice (13 out of 16) died before the projected endpoint of study. The original intention was that the mice would be sacrificed some days after the mice were injected with the xenografts. However, the mice were sacrificed at the time of death, which was after some days of injection. Therefore, it was possible that the mice were not given sufficient time to show any changes in tumor growth.

In subsequent studies, we could examine, at the molecular level, whether the PI3K/AKT/mTOR pathway was active in the control and whether it was, indeed, inhibited in the mice treated with RAD001. This analysis could be done by evaluating activity of the molecule mTOR and other downstream mTOR substrates. We could also determine the actual concentration of the androgens in the mice. The next step would be to evaluate the effect of increasing the androgens in the control group. This would be done by injecting a smaller number of androgen-dependent cells into each mouse, thereby ensuring that the mice would not be exposed to exogenous androgens.
of RAD001 within the experimental tissue. Perhaps the drug did not actually reach the target tissue or did not remain in the tissue long enough to be effective against prostate cancer cells. This could be a consequence of gavage as the route of delivery. Perhaps a more effective route of delivery would be injections of the drug at the site of the tumor or, alternatively, an implant near the tumor itself.

References