Establishment and Characterization of Transplantable, Luminescence Labeled Rat Renal Cell Carcinoma Cell Lines

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Purpose: Since renal cell carcinoma is considered an immunogenic tumor, testing therapeutic strategies has been impeded by the lack of relevant tumor models in immunocompetent animals. Recent advances in bioluminescence imaging permit sensitive in vivo detection and quantification of cells emitting light. Thus, we established bioluminescent rat renal cell carcinoma cell lines for immunocompetent rats.

Materials and Methods: The rat renal cell carcinoma cell line ACI-RCC stemming from chemically induced renal cell carcinoma in syngeneic ACI rats was stably transfected with a recombinant retroviral vector encoding luciferase genes derived from fireflies (ACI-RCC-ffLuc) or click beetles (ACI-RCC-cbLuc). Cell line growth patterns were characterized by bioluminescence imaging.

Results: Linear correlations noted observed between cell number and photon counts in each cell type. ACI-RCC-cbLuc emitted light about 500-fold higher than ACI-RCC-ffLuc. When transplanted subcutaneously, only ACI-RCC-ffLuc grew, possibly because of less antigenicity. ACI-RCC-ffLuc photon emission correlated significantly with subcutaneous tumor size. Orthotopic tumor growth and subsequent metastatic spread were monitored with time by increased photon intensity on bioluminescence imaging. Based on ACI-RCC-cbLuc bioluminescent intensity the in vitro screening test allowed the identification of several anticancer agents, including molecules related to human renal cell carcinoma progression.

Conclusions: The new in vivo rat renal cell carcinoma model with luciferase labeled tumor cells allowed us to monitor tumor growth noninvasively and semiquantitatively by bioluminescence imaging. This model system coupled with in vitro screening permits precise evaluation of tumor behavior in intact animals and determination of the therapeutic efficacy of anticancer agents for renal cell carcinoma.

Key Words: kidney; carcinoma, renal cell; luminescent proteins; cell line; models, animals

Renal cell carcinoma is a potentially lethal kidney disease. RCC treatment still depends on surgical resectability of the primary tumor but the prognosis in patients with metastatic disease remains poor.1 Antitumor cytokines such as interferon-α and IL-2 have been used widely to treat metastatic diseases but provided only limited therapeutic benefit.2 Thus, more effective treatments are needed for metastatic disease.

The advent of molecularly targeted therapy, eg bevacizumab, sorafenib,
sunitinib and temsirolimus, has improved the prognosis in patients with metastatic RCC, provided unprecedented response rates and significantly increased survival. However, complete tumor regression is sporadic and rare, and most patients ultimately have disease progression even during therapy. Further study is needed to identify more effective agents or combinations of targeted agents that can improve the objective response and hopefully lead to complete remission. Some questions have arisen, including whether there are appropriate therapeutic combinations to maximize efficacy with tolerable toxicity or multitargeted agents to inhibit different target molecules involved in cancer progression and whether it is possible to combine potential targeted agents with conventional cytotoxic chemotherapy or cytokine therapy.

To investigate the efficacy of novel agents or appropriate combinations of existing therapies in vitro and in vivo evaluations are required. Immunocompetent animals with normal immune systems are ideal since RCC is a representative immunogenic tumor. Among recent advances in small animal imaging BLI permits sensitive in vivo detection and quantification of cells specifically engineered to express Luc. BLI enables easy longitudinal quantification of the tumor burden in living animals. To date the Luc transfected murine RCC cell line RENCA, which is well characterized and arises spontaneously in Balb/c mice, is the only cellular source of the BLI model for in vivo study of RCC. However, using Luc labeled RENCA cells for in vitro determination is not well documented in the medical literature.

We established a new RCC cell line from immunocompetent rats and modified the cell lines for BLI. Two types of Luc expressing rat RCC cells were created. We report that cells engineered for BLI are available for in vitro culture and in vivo animal experiments. This model system also allows in vitro easy drug screening for potential effective compounds or appropriate drug combinations. Thus, we propose that this cell resource may be of great value to develop RCC therapy in humans.

MATERIALS AND METHODS

Animals and Cells
Six to 8-week-old male inbred ACI rats (CLEA Japan, Tokyo, Japan) were used. All experiments were conducted in accordance with the Jichi Medical University Guide for Laboratory Animals. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) with 10% heat inactivated fetal bovine serum under a humidified atmosphere with 5% carbon dioxide at 37°C.

Parental rat renal cancer cells (RCC), previously established from an ACI rat exposed to ferric nitrilotriacetate, were maintained in culture lethally irradiated NIH3T3 cells served as a feeder layer. Cells were cloned twice by limiting dilution in 96-well plates at a density of 0.1 cells per well and a homogeneous cell population was obtained. A total of 16 clones were isolated and clone 4 was used in subsequent experiments. Established cells, referred to as ACI-RCC cells, were grown in 10% fetal bovine serum-Dulbecco’s modified Eagle’s medium and used between passages 10 and 25.

Luc Transduced Cell Generation and Cell Growth Assay
Stable Luc expressing ACI-RCC cells (clone 4) were developed by transduction with a recombinant retroviral vector encoding neomycin resistance and fLuc or cbLuc. Plasmids were derived from pMEI-5Neo (Takara Bio, Shiga, Japan) and pGL3 (Promega) for fLuc and pELuc (Toyobo, Osaka, Japan) for cbLuc. ACI-RCC cells were infected with the supernatant from the 293 producer cells in the presence of Polybren® (8 μg/ml). Transduced cells were selected with G418 gentamicin (Invitrogen™) (500 μg/ml). Generated colonies were screened using a Luc assay. To determine bioluminescence activity cells were seeded at a serial concentration in triplicate in 96-well plates and measured using the IVIS™ bioimaging system with the Bright-Glo™ Luciferase Assay System as a substrate according to the manufacturer protocol. Transduced ACI-RCC-fLuc and ACI-RCC-cbLuc cells were propagated separately in medium containing G418 (500 μg/ml). Cells (1 × 10⁵) were incubated for 96 hours. The total viable cell number was determined by the trypan blue exclusion test every 24 hours using a hemocytometer.

Bioluminescence Imaging
In vivo Luc imaging was done with the noninvasive IVIS in vivo imaging system. One minute after intravenous injection of D-luciferin (30 mg/kg) (potassium salt, Biosynth, Postfach, Switzerland) the signal intensity of emitted photons from Luc expressing tissue was measured with a 1-minute integration time. For ex vivo detection tissues were procured after intraperitoneal injection of D-luciferin (60 mg/kg) and immersed in D-luciferin (300 μM in phosphate buffered saline) for measurement by a Luc assay. Living Image® software was used to analyze the data.

Tumor Models
For the subcutaneous tumor model tumor cells (1 × 10⁶) were injected into the abdominal subcutaneous space in rats. Tumor volume was evaluated using the equation, tumor volume = (length in mm) × (width in mm)²/2. For the orthotopic kidney tumor model cells (1 × 10⁶) were suspended in phosphate buffered saline (0.1 ml) and inoculated into the left renal subcapsular space. Obvious tumor was established about 1 week later.

In Vitro Sensitive Compound Screening
The SCADS inhibitor kit comprises 3, 96-well plates, including 288 chemicals that target definitive molecules previously established as inhibitors. Each compound was provided at 2 mM in DMSO in each well with 5 μl. To screen compounds sensitive to ACI-RCC cells ACI-RCC-cbLuc cells were seeded at a density of 2 × 10³ in 3, 96-well plates before adding each compound to a final volume of 10 μM. Preparations were incubated for 48 hours. Luminescence intensity was deter-
mined by a luminometer and calculated as a proportion vs the DMSO control alone. An intensity proportion of less than 15% was considered sensitive.

Statistical Analysis
The Student t or Mann-Whitney U test was used to determine p values using StatView® software. Data are expressed as the mean ± SD. Differences between groups were considered significant at p < 0.05.

RESULTS
ACI-RCC Line Characteristics. To permit growth in culture ACI-RCC cells were cultured in the presence of lethally irradiated feeder NIH3T3 cells. A total of 16 clones were isolated and clone 4 was maintained in culture without feeder cells. Thereafter ACI-RCC clone 4 was established as an ACI-RCC line, as described.

To examine whether the established cell line could be transplantable to ACI rats cells (1 × 10⁶) were injected into the subcutaneous space of ACI rats. Visible tumor formed 7 days after tumor inoculation and the tumor growth pattern was equivalent to that of original parent cells, which could only survive in vivo (fig. 1, A). Microscopy of the established tumor revealed moderately differentiated carcinoma of the basophilic cell type (fig. 1, B). When cells were implanted orthotopically beneath the renal capsule in ACI rats, metastatic spread to the lung also occurred around 2 weeks later (fig. 1, C). The animals died 3 to 4 weeks after tumor inoculation.

Establishment. Recent advances in BLI technology have facilitated quantitative analysis of cellular processes in vivo. ACI-RCCs were transduced with ffLuc or cbLuc to visualize tumor progression in vivo. The advantage of using Luc as a marker includes its sensitivity in vitro and its linear dose dependent output of light in the presence of D-luciferin (fig. 2). The photon intensity of ACI-RCC-cbLuc cells was about 500-fold higher than that of ACI-RCC-ffLuc cells at the same cell number. Each cell line had a similar cell proliferation pattern and cell morphology (fig. 3).

ACI-RCC-ffLuc and ACI-RCC-cbLuc BLI
To investigate ACI-RCC-ffLuc and ACI-RCC-cbLuc cell tumor formation cells (1 × 10⁶) were subcutaneously implanted and tumor growth was monitored by BLI and tumor volume measurement. Tumors formed

Figure 1. A, in vivo growth kinetics of subcutaneous tumor model of original parental cells in vivo and ACI-RCC parental cells in 8 rats each show no difference in in vivo tumor growth rate in 2 cells types. B, representative histology of established kidney subcapsular tumor 7 days after tumor cell implantation. Dotted line indicates parenchymal border. H & E, reduced from ×40. C, representative histology of lung metastatic foci on day 14. H & E, reduced from ×40.

Figure 2. One of 2 independent experiments with similar results shows correlation between cell number and photon intensity using BLI. Cells were seeded at indicated density in triplicate wells and imaged after adding Luc. Mean photon intensity was plotted against cell number per well. Number of cells correlated with amount of emitted light above detection limit. A, ACI-RCC-ffLuc cells with 1 × 10⁴ detection limit (r = 0.997, p < 0.001); B, ACI-RCC-cbLuc cells with 1 × 10⁵ cell detection limit (r = 0.993, p < 0.001). g, gm.
about 1 week after tumor injection of parent ACI-RCC and ACI-RCC-ffLuc cells but no tumors formed after ACI-RCC-cbLuc cell injection. BLI revealed substantial Luc derived photon counts at the implanted site of ACI-RCC-ffLuc and ACI-RCC-cbLuc cells immediately after tumor implantation. Luc activity of ACI-RCC-ffLuc cells generally attained a nadir 24 hours after inoculation and increased to detectable levels by day 7, when tumor was visible (data not shown). However, the strong photon emission from ACI-RCC-cbLuc cells observed for 1 week after injection suddenly vanished at day 9 and tumors of ACI-RCC-cbLuc cells did not form (fig. 4, A). The difference in in vivo growth behavior between ACI-RCC-ffLuc and ACI-RCC-cbLuc cells may be due to the antigenicity of cbLuc protein for immunocompetent rats since ACI-RCC-cbLuc cells grew in xenogenic athymic mice (fig. 4, B). Results show that ACI-RCC-ffLuc cells are available for analysis of BLI mediated RCC dissemination.

Transduction of the flLuc gene did not affect the growth kinetics of subcutaneous tumors (fig. 5, A). There was significant correlation between tumor size and photon intensity (r² = 0.9825, p < 0.001, fig. 5, B). Results indicate the usefulness of this Luc labeled tumor model system as a quantitative tool for growing tumor lesions.

We further evaluated orthotopic tumor growth and metastatic spread using this model. Photon emission was detectable over the tumor site at day 10 and attained a plateau around 3 weeks after orthotopic tumor implantation (fig. 6, A). BLI visualized tumor metastasis to the lung about 2 weeks after implantation and photon counts also increased at metastatic sites (fig. 6, B). Ex vivo luminescence analysis showed that the tumor burdened kidney and the metastasized lungs in rats were substantially Luc positive (fig. 6, C).

**Easy In Vitro Screening of Compounds Sensitive to ACI-RCC Cells**

While ACI-RCC-cbLuc cells were potentially immunogenic in immunocompetent ACI rats, the strong photo emission potential may allow sensitive drug screening. To address this we investigated whether ACI-RCC-cbLuc cells are available for in vitro drug screening. ACI-RCC-cbLuc cells (2 × 10³) were plated on 96-well plates before the compound was added and then incubated for 48 hours. Luminescence intensity was simply determined by a luminometer and calculated as a proportion vs the DMSO control alone. Of the 288 compounds arrayed in the SCADS inhibitor kit 21 showed decreased

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**Figure 4.** BLI monitoring of subcutaneously implanted ACI-RCC-cbLuc cells. A, 1 of 2 independent experiments in 5 rats each with similar results shows abolished strong photon emission 9 days after cell implantation with tumor formation never confirmed. g, gm. B, representative BLI of ACI-RCC-cbLuc cells 10 days after cell implantation in Balb/c-nu/nu mouse reveals prominent photon emission from established tumor site.
photons with less than 15% considered to have inhibitory effects on ACI-RCC cells with control considered 100% (see table). These data suggest that this RCC cell line is useful as a cell resource for easy drug screening. Notably some candidate compounds targeting molecule heat shock protein 90, AKT, Met and platelet-derived growth factor have been used in the clinical setting in patients with RCC.

**DISCUSSION**

Increased knowledge of cancer progression and therapeutic resistance has identified many gene products involved in apoptosis, proliferation and other vital cell functions. Novel agents directed at these targets have been developed and show promise in vitro but have not always been consistent in vivo. Because RCC is considered an immunogenic tumor, a reliable and quantifiable orthotopic animal model of RCC in normal immune systems is relevant. To our knowledge murine RCC (RENCA) is the only animal model of RCC in immunocompetent syngeneic Balb/c mice. It represents reliable, reproducible growth characteristics and has a metastatic pattern similar to that of RCC observed clinically in humans. However, its further use for biomedical research, which requires blood and tissue sampling or treatment models using surgical manipulation, may be restricted due to limitations imposed by body size. Thus, we established the rat RCC cell line derived from a ferric nitrilotriacetate induced renal tumor and in vivo tumor models in immunocompetent ACI rats, providing another useful model in which to study RCC. We also generated Luc expressing cells, which enabled in vivo monitoring of cancer progression by BLI, the most commonly used modality for noninvasive imaging in small animals. Of Luc genes widely used as light emitting reporters we used 2 types, including that of Phototinus pyralis (firefly) and Phototinus plagiophthalamus (click beetle). Each

**Figure 5.** BLI monitoring of subcutaneously implanted ACI-RCC-ffLuc cells. A, in vivo growth kinetics of subcutaneous tumor model of parental and ACI-RCC-ffLuc cells revealed no difference between cells in vivo tumor growth. Each experimental group had 8 rats. B, bioluminescence intensity and tumor volume correlated significantly. g, gm.

**Figure 6.** BLI monitoring of orthotopically implanted ACI-RCC-ffLuc cells. A and B, 1 of 2 independent experiments with similar results. Two of 5 rats in each experimental group died on day 22. g, gm. A, mean photon intensity of subcapsular tumor with time. BLI shows tumor growing in renal subcapsular space. B, mean photon intensity of lung metastatic lesion with time. Representative BLI reveals subcapsular renal tumor (arrows) and lung metastatic lesion (circle). C, ex vivo analysis reveals subcutaneous (a) and subcapsular (b) tumors, and lungs (c).
Luc can catabolize a substrate D-luciferin, which is relatively stable in vivo. Using the newly established bioluminescent rat RCC cell line we developed a novel system to test in vitro and in vivo tumor growth.

We first examined the in vitro characteristics of 2 Luc labeled ACI-RCC cell lines. ACI-RCC-cbLuc cells were superior to ACI-RCC-ffLuc cells for in vitro measurement in terms of luminescence intensity with a much lower detection limit. As reported in previous studies of other tumor models, the number of tumor cells in vitro correlated with the bioluminescence signal, indicating the quantitative value of this system for in vitro measurement (fig. 2). This appeared reproducible in solid tumors but not in an IL-3 dependent murine leukemia cell line, in which signal intensity was affected by IL-3 concentration and proliferation stage.12–14

We then examined in vivo the growth of these luciferase labeled ACI-RCC cell lines. ACI-RCC-cbLuc cells were rejected completely by syngeneic ACI rats while ACI-RCC-ffLuc cells were consistently taken to establish tumors. This difference may be due to the higher antigenicity of Luc protein derived from Phototinus plagiophthalamus, which predisposes ACI-RCC-cbLuc cells to increased susceptibility to attack by the host cellular immune defense system. This susceptibility may be explained by the fact that ACI-RCC-cbLuc cells grew in athymic mice. Even with syngeneic transplant-plantation transplanted cells expressing a marker protein such as green fluorescent protein and β-galactosidase (LacZ) occasionally disappear due to immunogenicity.15,16 Even flLuc, which is considered less immunogenic than green fluorescent protein,17 has been reported to induce a T-helper type 1 immune response after intradermal immunization in immune competent Balb/c mice.18

These factors may subsequently raise the question of the difference in antigenicity between ACI-RCC-ffLuc and ACI-RCC-cbLuc cells. The quantity or quality of Luc protein expressed in each cell line must be considered, although it is hard to accurately compare the differences. We assume that the former is less likely to be the reason for the different fates in vivo of the 2 cell lines. This assumption is supported by the finding that enhanced flLuc expression in mouse T cells permitted sensitive tracking of adoptively transferred T cells infiltrating tumor sites without increasing immunogenicity in immunocompetent murine models.19 Thus, immune responses involving the different Luc proteins may have to be considered cautiously. Thereafter we focused on ACI-RCC-ffLuc cells to investigate in vivo tumor growth using an in vivo imaging system.

A significant correlation between luminescence intensity and subcutaneous tumor volume verified the quantifiable nature of our Luc labeled tumor model system (fig. 5, B). Because of the impossibility of measuring tumor size using calipers in orthotopic RCC models, therapeutic experiments are usually assessed by animal symptoms and end point variables, such as survival time, kidney weight and histological analysis of explanted kidneys. However, our system enabled noninvasive real-time extracorporeal monitoring of orthotopic tumors beneath the renal capsule. Notably subsequent metastasis of the orthotopic tumor to the lung could also be monitored (fig. 6, B). This metastatic pattern, similar to the RENCA model, mimics that in human RCC, which is clinically meaningful for studying RCC progression.

Our use of rats rather than mice was also advantageous for BLI. Easy intravenous delivery of the substrate D-luciferin enabled rapid, stable measure-
ment of bioluminescence intensity. Photon counts measured by IVIS peaks 1 minute after intravenous injection of D-luciferin had fewer individual differences in our models (data not shown). The window of reliable maximum photon emission in murine models requiring intraperitoneal injection of Luc varies among studies and is reportedly much longer, usually 15 minutes or more.12,20,21

Finally, we examined the use of our Luc labeled rat RCC cell line ACI-RCC-cbLuc as an in vitro screening tool for anticancer agents. The results of drug sensitivity using the SCADS inhibitor kit combined with Luc labeled cells provided a useful screening tool with rapid high throughput. Screened compounds with an inhibitory effect on ACI-RCC cells included molecules related to RCC progression, ie heat shock protein 90, AKT, Met and platelet-derived growth factor, which are molecular targets of recent RCC therapies (see table).12,22,23 These results imply the practical accuracy and feasibility of our systems for in vitro RCC investigation. The exact efficacy and toxicity of such screened compounds in vivo as well as appropriate combinations are under investigation at our laboratory.

CONCLUSIONS

We engineered Luc expressing cancer cells from a newly established rat RCC cell line arising from immunocompetent rats, and noted easy quantitative detection of orthotopic tumor growth and metastatic spread in real time using the Luc based bioluminescent systems. We also introduced a feasible method of in vitro screening for anticancer compounds using Luc labeled rat RCC cells. This Luc based in vivo model system coupled with the in vitro screening system may facilitate the development of new therapies that may eradicate metastatic RCC. Also, our allograft tumor model in an immunocompetent animal may be advantageous for studying exact treatment efficacy and safety for immunogenic tumors such as RCC.

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REFERENCES