Induction of Tumors in Heterotopic Bladder by Topical Application of N-Methyl-N-nitrosourea and N-Butyl-N-(3-carboxypropyl)nitrosamine

Ryoichi Oyasu, Takuo Iwasaki, Michio Matsumoto, Yoshihiko Hirao, and Yoshiki Tabuchi

Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611

ABSTRACT

The heterotopic urinary bladder with a communicating reservoir is a potentially useful model for bladder carcinogenesis studies. As a test of whether such bladders will develop transitional cell carcinomas after chronic carcinogenic stimuli, two carcinogens, N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)nitrosamine, were instilled repeatedly into the reservoir connected with the heterotopic bladder. Transitional cell carcinomas developed in 25 of 33 heterotopic bladders exposed to cumulative doses of 1.5, 3.0, or 6.0 mg of N-methyl-N-nitrosourea for between 20 and 30 weeks, while heterotopic bladders exposed to cumulative doses of 150 or 300 mg of N-butyl-N-(3-carboxypropyl)nitrosamine failed to develop tumors. However, 11 of 27 rats with heterotopic bladders that were exposed to N-butyl-N-(3-carboxypropyl)nitrosamine for over 20 weeks developed tumors in their homotopic or natural bladders. N-Methyl-N-Nitrosourea probably acted directly on the bladder epithelial cells to induce neoplastic change. The reason(s) for the development of tumors in homotopic but not heterotopic bladders when N-butyl-N-(3-carboxypropyl)nitrosamine was administered directly into the heterotopic bladders could not be ascertained from these studies.

INTRODUCTION

A good working model for urinary bladder carcinogenesis studies should meet the following requirements: (a) the test site should be lined by a transitional epithelium; (b) it should be free from infection or calculus formation; (c) it should retain the instilled carcinogen for a reasonable period of time; (d) it should be possible to instill test chemicals into the lumen easily and repeatedly; and (e) the epithelium should not be exposed to the urinary metabolites of the test compound. In the search for such a model, attempts to transplant bladder tissue and induce tumors in such tissue were reported previously (14, 15, 17, 18). The heterotopic bladder with a communicating reservoir was developed in our laboratory as a potentially useful model for bladder carcinogenesis studies. As a test of whether such bladders will develop transitional cell carcinomas in response to an appropriate carcinogenic stimulus, this report describes induction studies in the heterotopic bladder following repeated instillation of carcinogenic compounds into the lumen of the communicating reservoir.

MATERIALS AND METHODS

General

Young male and female CD Fischer rats (Charles River Breeding Laboratory, Wilmington, Mass.) weighing 130 to 180 g were used as both donors and recipients. For the MNU study, male rats only were used, and for the BCPN study female rats only were used. Transplantation was performed always between the same sex. Ommaya side-inlet flushing reservoirs (Model No. 850-1274; Heyer-Schulte Corp., Goleta, Calif.) with an attached cannula were prepared as previously described (13). MNU (ICN Pharmaceuticals Inc., Life Sciences Group, Plainview, N. Y.) was divided into 1-g portions and stored frozen at −20° until use. BCPN was purchased from Izumi Chemical Laboratory, Yokohama, Japan.

Preparation of Animals and Surgical Procedures

The procedure for transplantation of urinary bladder has been described in detail (13). Shortly after the investigation began, a high incidence of infection of the reservoir-bladder unit occurred, and the animals were forced to be discarded. Investigation of the source of infection disclosed 2 probable sources; the first was the bacteria grown on culture of urine collected by bladder puncture from as many as 25% of healthy CD Fischer rats. The second was the dermal bacteria introduced during the operative procedure due to inadequate disinfection of the skin. Subsequently, the following...
modifications were made: (a) 18 hr before transplantation, donor rats received a single i.m. injection of 4 mg gentamicin sulfate (Schering Corp., Kenilworth, N. J.); (b) for preparation of the operative fields, the skin was first painted with Betadine (Purdue Frederick Co., Norwalk, Conn.), and after 3 to 4 min it was washed with 70% ethanol; (c) after the bladder-reservoir unit was affixed tight to the fascia by several sutures, 1 mg of gentamicin in 0.1 ml of 9% NaCl solution was instilled into the reservoir; and (d) finally to prevent infection, gentamicin was always added to the injection solution at the concentration of 1 μg/ml. A second source of earlier failure cases was loss of function of the unit as a result of marked contracture of the transplants due to fibrosis and/or to occlusion of the cannula tip by an inflammatory polyp. In an attempt to reduce such failure cases, the transplanted bladders were kept distended to a 1-cm diameter with 0.6 to 0.7 ml of 9% NaCl solution to prevent possible formation of an inflammatory polyp due to mechanical irritation by the tip of the cannula.

One week after transplantation, patency of the cannula was tested by flushing the reservoir with 0.9% NaCl solution with a tuberculin syringe. If the transplant was less than 1 cm in diameter, 0.9% NaCl solution was added to inflate it to that size. The aspirate was cultured by streaking on a blood agar plate. If the culture was positive for bacteria, 1 mg of gentamicin dissolved in 0.9% NaCl solution was added to the reservoir at weekly intervals for several times. However, as more experience was gained, animals with an infected reservoir-bladder unit were discarded because infection, if once occurred, frequently resulted in necrosis of the system.

Experimental Groups

MNU and BCPN were chosen as test compounds because both were shown to be carcinogenic to the bladder mucosa following intravesicular instillations into intact natural bladders (4, 5).

MNU Administration into the Reservoir-Bladder Unit.

For this study, male rats only were used. Three dose levels used were 0.25, 0.5, and 1.0 mg each once every 2 weeks for 6 doses. The carcinogen solution was prepared fresh each time by dissolving it in 0.9% NaCl solution. It was passed through 0.45-μm Millipore filter membrane (Millipore Corp., New Bedford, Mass.) before use.

Administration of MNU was initiated 2 weeks post-transplant. Following aspiration of 0.1 to 0.2 ml reservoir content, the scheduled dose of MNU in 0.2 ml of 0.9% NaCl solution was injected into the reservoir. Depending upon the size of the bladder, 0.9% NaCl solution was also added to make the bladder diameter approximately 1 cm. Following injection of these solutions, the reservoir-bladder system was flushed by aspirating the content into syringe several times. The function of the reservoir system was checked biweekly between injections of MNU by flushing the reservoir with 0.9% NaCl solution. Patency of the cannula was confirmed by palpating the tension of the transplanted bladder. Following completion of carcinogen injections, the reservoir received a weekly injection of 0.3 ml of 0.9% NaCl solution. The control group animals received a weekly injection of 0.9% NaCl solution throughout the experiment. The experiments were terminated 30 weeks after the first MNU injection.

BCPN Administration into the Reservoir-Bladder Unit.

For this study, female rats were used so that the results could be compared with those obtained from a similar study with homotopic bladders (unpublished data). Two dose levels were used, 2.5 and 5.0 mg BCPN 3 times weekly for 20 weeks followed by a weekly injection of 0.9% NaCl solution for an additional 10 weeks. This schedule was generally patterned after a similar study by Hashimoto et al. (4). The animals receiving 5 mg of BCPN per injection consisted of 2 groups: in Group 1, BCPN treatment was delayed until 2 weeks after transplantation by which time reepithelialization of the bladder mucosa was expected to be complete; in Group 2, BCPN treatment was initiated 5 to 6 days posttransplant when active regeneration of bladder epithelium was in progress. The sterile 0.9% NaCl solution containing BCPN after pH had been adjusted to 6.5 by NaOH was made fresh every 2 to 4 weeks and was stored refrigerated until use. The control group animals received 0.3 ml of 0.9% NaCl solution 3 times weekly for 20 weeks and thereafter once a week for an additional 10 weeks. The experiment was terminated at 30 weeks.

Removal and Examination of Bladder

The animals were killed with an overdose of ether, and their weights were recorded. A complete autopsy examination was performed. The reservoir-bladder unit was removed. Function of the reservoir was checked. The bladder was then carefully separated from the cannula, opened along its long axis, stretched on a piece of cardboard with pins, and inspected for gross changes under a dissecting microscope. The homotopic bladder was removed and inspected likewise under a dissecting microscope.

Histological Examination

Following 24 hr fixation in 10% neutral formalin, the bladder was cut into 3 pieces along the long axis, and all were submitted for microscopic examination. Sections were taken from 3 different levels and stained with hematoxylin and eosin.

The normal heterotopic bladder mucosa consisted of a 3-cell layer-thick transitional epithelium (Fig. 1). If the epithelium was more than 4 cell layers thick, it was referred to as simple hyperplasia. Extent of hyperplastic foci varied from animal to animal. When hyperplastic epithelium formed a finger-like excrescence toward the lumen, the changes were regarded as papillary hyperplasia (Fig. 3). These papillae were supported by a delicate fibrovascular stroma that showed no branching. If the localized proliferation was predominantly downwards, the changes were classified as nodular hyperplasia (Fig. 4). Frequently, nodular and papillary hyperplasias coexisted in the same bladder.

Neoplasms were divided into 4 stages, 0, A, B, and C, depending upon the depth of invasion, following the classification by Jewett and Strong (8). The tumor cells were divided into 3 grades depending upon the degree of anaplasia. Grade 1 carcinomas (Figs. 9 and 10) consisted of transitional cells with minimal atypia; the nuclei were vesicular and larger than those of normal cells but uniform in
size and shape. Nucleoli were indistinct, and mitotic figures were virtually absent. The nuclei of Grade 2 carcinomas (Figs. 7 and 8) were more pleomorphic, and the nucleoli were prominent. Mitotic figures were readily detected. Grade 3 carcinomas (Figs. 11 and 12) were characterized by marked cytological and architectural abnormality. Nuclear pleomorphism was marked, and mitotic figures were common. It was in this group of carcinomas that the malignant squamous cell component was frequently observed. Almost all carcinomas were nodular to nodulopapillary in microscopic appearance. No metastasis was detected.

An inflammatory polyp (Figs. 5 and 6) was defined as a polypoid intraluminal protrusion with a broad stalk. They were located in the mucosa either opposite to or near the tip of the cannula. Under a dissecting microscope, the surface was hemorrhagic or hyperemic. Microscopically, the surface was covered by a hyperplastic epithelium commonly showing focal squamous metaplasia (Fig. 6). The underlying stroma was either edematous or fibrous, often containing granulation tissue and chronic inflammatory cells. Because of its location and the characteristic appearance, it was not difficult to distinguish the inflammatory polyp from neoplastic lesions that may likewise be polypoid.

RESULTS

General

Altogether, 207 rats received bladder transplants, of which 102 rats carried sterile “functioning” bladders through the 30-week experimental period. Three additional rats were killed between 20 and 30 weeks because in 1 rat (MNU, 1.0 mg for 6 doses) repeated aspirations from the reservoir-bladder unit were hemorrhagic and contained atypical cells suspicious of cancer. Two other rats were killed because of loosening of connection between the reservoir and the connector (MNU, 0.25 mg for 6 doses) and chewing of reservoir (BCPN, 5 mg, 3 doses/week).

The animals gained weight progressively, and there was no significant weight difference between the experimental and control group.

Two major causes of 102 earlier failure cases were infection of the reservoir-bladder unit (40 rats) and loss of function of the unit as a result of marked contracture of the transplants due to fibrosis and/or occlusion of the cannula tip by an inflammatory polyp (53 rats). Both complications occurred predominantly during the initial phase of the study and prompted modifications of the operative procedures as described earlier. The protective measures such as a preoperative i.p. injection of gentamicin, strict preoperative dermal disinfection, and distension of the heterotopic bladder with 0.9% NaCl solution reduced such complications significantly.

No stone or parasite was detected in either homotopic or heterotopic bladders.

Pathological Changes in MNU-treated Heterotopic Bladders

The incidence of hyperplasia and tumors in MNU-treated heterotopic bladders is shown in Table 1. Simple hyperplasia 4 to 5 cells thick was observed in almost all bladders. Extent of hyperplasia varied from case to case. Nodular and/or papillary hyperplasia was found always in association with simple hyperplasia. Transitional cell carcinomas were observed in heterotopic bladders exposed to all 3 dose levels. In 18 of the 25 cases, the tumors were multiple. With the lowest dose of MNU (0.25 mg for 6 doses), tumors were found in 7 of 12 bladders, and these tumors tended to be of low-grade cancer. No histological evidence of invasion was observed with this dose level. With the intermediate dose level of MNU (0.5 mg for 6 doses), all of the 11 heterotopic bladders that survived over 20 weeks developed tumors, several of which were invasive (Figs. 7 and 8). One of the tumor-bearing animals was sacrificed at 20 weeks because a repeated cytological examination of brown aspirates contained atypical cells highly suspicious of cancer. Histological examination of the resected heterotopic bladder revealed a Grade 2, Stage A carcinoma. The homotopic bladders were all normal.

Pathological Changes Associated with BCPN Administration Into Heterotopic Bladders

No tumors developed in these heterotopic bladders (Table 2; Fig. 2). However, 11 of 27 rats that received a higher dose of BCPN in their heterotopic bladders developed transitional cell carcinomas in their homotopic or natural bladders (Figs. 9 and 10), and 8 of these were multiple.

Since the starting date of BCPN treatment (2 weeks versus 5 days posttransplant) did not affect the incidence of proliferative lesions in either heterotopic or homotopic
Pathological changes in the heterotopic and homotopic bladders following repeated administration on BCPN into the reservoir connected with the heterotopic bladder (female Fischer rats)

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>BCPN 2.5 mg 3 times/wk</th>
<th>BCPN 5.0 mg 3 times/wk</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>30</td>
<td>66</td>
<td>26</td>
</tr>
<tr>
<td>&gt;20 wk</td>
<td>12</td>
<td>27 (p &lt; 0.05)</td>
<td>16</td>
</tr>
<tr>
<td>Histopathology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal epithelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterotopic</td>
<td>5a</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Homotopic</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Hyperplasiaa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simple</td>
<td>7</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Nodular</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Papillary</td>
<td>1</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Tumorb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stage 0</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Stage A</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stage B</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Grade 1</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Grade 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiple</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Inflammatory polyp</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fibrosis of lamina propria</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Number of animals showing respective histopathological change. For tumor classification only, the most advanced stage or grade, if tumors were multiple, was used.
b For the definition of hyperplasia, tumor stage, and grade, refer to the text.

R. Oyasu et al.

The results indicate that the heterotopic bladders develop carcinomas in response to MNU. Although all 3 dose levels were tumorigenic, there was a tendency for those carcinomas associated with the lowest level (1.5 mg of cumulative dose of MNU) to be low-grade malignant. All were noninvasive. On the other hand, the heterotopic bladders failed to develop tumors following repeated topical applications of BCPN. Of particular interest is the fact that 11 of 27 homotopic bladders developed transitional cell carcinomas when BCPN was repeatedly instilled into the reservoir of the heterotopic bladders. The tumors were multiple in 8 animals and invasive in 5. The reason(s) for the development of tumors in homotopic but not heterotopic bladders when BCPN was administered directly into the heterotopic bladders could not be ascertained from these studies. It is possible that BCPN per se may not be a bladder carcinogen. After being absorbed through the wall of the heterotopic bladder, it may undergo metabolic activation in the liver. The metabolites of BCPN may then be excreted into the urine and conceivably exert carcinogenic action on the homotopic bladder. It has been suggested by Okada et al. that BCPN is a proximate carcinogen, based on: (a) the observation of in vitro malignant transformation of rat bladder cells after chronic treatment with BCPN and urea but not with BCPN alone; (b) the fact that BCPN is the direct-acting carcinogen with a proven bladder carcinogenicity when applied topically (5), and BCPN, which is a metabolite of N,N-dibutylaminoamine and is believed to be a proximate carcinogenic metabolite active on urinary bladder epithelium (3, 4, 9, 10).
major urinary metabolite in the susceptible species (the rat) following p.o. or s.c. administration of N,N-dibutylnitrosamine, N-butyl-N-(4-hydroxybutyl)nitrosamine, or BCPN (9); and (c) induction of bladder tumors by Hashimoto et al. (4) after repeated instillation of BCPN into the bladder of rats. In the intravesicular instillation experiment by Hashimoto et al. (4), a high incidence of calculi was encountered including the presence of calculi in 3 bladders that developed tumors. With the schedule of Hashimoto et al., induction of bladder tumors by repeated intravesicular instillation of BCPN was attempted in our laboratory. Of 12 rats that survived more than 20 weeks, 8 developed calculi in their bladders, and all had diffuse papillomatosis. The remaining 4 rats that were free of calculi showed no tumors but did have simple hyperplasia. A high incidence of vesical calculi was also observed in the control animals, and all 4 such rats revealed diffuse papillomatosis. No tumor developed in the control rats that were free of calculi (unpublished data).

Accordingly, the results of these studies on the BCPN administration into the natural or homotopic bladders should not be construed as evidence that BCPN is a proximate carcinogen in view of the concurrent development of calculi. However, the possibility that BCPN may be a direct-acting bladder carcinogen that requires urine or urinary components to express its carcinogenicity cannot be excluded. The absence of urine in the heterotopic bladders may in part be responsible for the lack of development of tumors in these bladders exposed to BCPN.

It is also possible that, although the heterotopic bladder mucosa is morphologically normal or nearly normal at ultrastructural as well as light-microscopic levels, it might be biologically different from that of the homotopic bladder. The epithelial cells may be unable to activate BCPN to ultimate carcinogen(s), whereas this property may be inherent in the homotopic or natural bladder.

Finally, sex difference could account for the failure of tumor development in the heterotopic bladders following direct application of BCPN since female rats appear to be less susceptible than are male rats to chronic p.o. administration of N-butyl-N-(4-hydroxybutyl)nitrosamine, a compound closely related to BCPN (11). It is now clearly established that following long-term administration of various bladder carcinogens the bladder mucosa undergoes a series of proliferative changes before invasive tumors develop (1, 6, 7, 16). The earliest change is simple or orderly hyperplasia followed by nodular and/or papillary hyperplasia and finally by invasive carcinomas. Occurrence of this histological sequence was well demonstrated in this study in both the heterotopic and homotopic bladders.

The 2 major complications, infection and loss of function of the reservoir-bladder unit, were serious problems during the initial phase of our study, and as a result, a large number of recipients were forced to be killed. With the introduction of the protective measures, both complications were greatly reduced. Currently, our success rate to carry "functional" bladders beyond 20 weeks is approximately 80%. With flushing the reservoir-bladder unit once a week, the heterotopic bladder can be maintained as functional for an indefinite period of time. The only complication that may still develop despite the various protective measures is ischemic contracture of the transplants, which involves approximately 10% of the transplants. These animals must be removed from the study groups. Possible importance of the mild fibrosis commonly observed in the lamina propria of the heterotopic bladder (Fig. 2) must be taken into account. Altered epithelial-mesenchymal interaction as a result of fibrosis might be responsible for the difference in the epithelial response to carcinogen.

We believe that the heterotopic bladder described here is a useful model for bladder carcinogenesis studies. It would be particularly useful to assess the carcinogenicity of reactive unstable compounds and to study the role of chemopreventive and cocarcinogenic substances.

ACKNOWLEDGMENTS

Dr. Denise Hidvegi kindly performed cytological examination of the aspirates from the reservoir-bladder unit.

REFERENCES


Fig. 1. Heterotopic urinary bladder, 30 weeks posttransplant, in a female rat. The bladder received 0.9% NaCl solution 3 times weekly for 20 weeks and thereafter once a week for an additional 10 weeks. H & E, × 200.

Fig. 2. Heterotopic bladder, 30 weeks posttransplant, that was exposed to cumulative dose of 300 mg BCPN. The epithelium is normal. Lamina propria shows mild degree of fibrosis. H & E, × 200.

Fig. 3. Papillary hyperplasia observed in heterotopic bladder, 30 weeks posttransplant, that was exposed to cumulative dose of 1.5 mg MNU. The papilla is supported by a delicate fibrovascular stroma that shows no branching. H & E, × 100.

Fig. 4. Nodular hyperplasia observed in the heterotopic bladder shown in Fig. 3. H & E, × 100.

Figs. 5 and 6. Inflammatory polyp observed in a heterotopic bladder, 30 weeks posttransplant, that was exposed to cumulative dose of 300 mg BCPN. It is covered by a hyperplastic epithelium showing squamous metaplasia. Focally granular cell layer has been formed. The stroma is loosely fibrous and contains dilated capillaries. Fig. 5, H & E, × 30; Fig. 6, H & E, × 200.
Induction of Tumors in Heterotopic Bladder

Figs. 7 and 8. Transitional cell carcinoma Grade 2 and Stage B observed in a heterotopic bladder, 30 weeks posttransplant, exposed to cumulative dose of 3.0 mg MNU. It is a nodulopapillary exophytic lesion rich in vascular stroma. The tumor cells have oval to round nuclei with a prominent nucleolus. Mitoses are occasionally observed. The tumor has invaded the tunica muscularis. The invasive front shown in Fig. 7 (lower center) is magnified in Fig. 8. Two muscle bundles have been replaced partially by tumor cells. The stroma contains a moderate number of chronic inflammatory cells, mostly lymphocytes and macrophages. Fig. 7, H & E, x 50; Fig. 8, H & E, x 200.

Figs. 9 and 10. Transitional cell carcinoma Grade 1 and Stage 0 observed in a homotopic bladder of the rat whose heterotopic bladder was exposed to cumulative dose of 300 mg BCPN 30 weeks posttransplant. Tumor cells grown in nodulopapillary pattern are supported by branching delicate fibrovascular stroma. The nuclei are larger than normal but uniform in size and shape. Fig. 9, H & E, x 30; Fig. 10, H & E, x 200.

Figs. 11 and 12. Transitional cell carcinoma Grade 3 and Stage A observed in a heterotopic bladder, 30 weeks posttransplant, exposed to cumulative dose of 6 mg MNU. It is a broad-based typical papillary carcinoma composed of large pleomorphic cells showing squamous metaplasia focally. The nuclei are pleomorphic, and the nucleoli are prominent. Fig. 11, H & E, x 30; Fig. 12, H & E, x 200.

Downloaded from cancerres.aacrjournals.org on January 12, 2018. © 1978 American Association for Cancer Research.
Induction of Tumors in Heterotopic Bladder by Topical Application of $N$-Methyl-$N$-nitrosourea and $N$-Butyl-$N$-(3-carboxypropyl)nitrosamine

Ryoichi Oyasu, Takuo Iwasaki, Michio Matsumoto, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/38/9/3019

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/38/9/3019. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.