JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2015. Vol. **9**(3), p. 2379-2384

Matrix Metallo Proteinases Activities in N-methyl-N-nitrosourea Induced Mammary Tumour in Wistar Rats

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(Received: 11 April 2015; accepted: 15 June 2015)

Female Wistar rats of 50 days old were administered with five doses of 50 mg/kg body weight N-methyl-N-nitroso Urea (MNU) intraperitoneally at weekly intervals. Animals were physically examined for tumour induction twice weekly throughout a 25 week observation period. Both malignant and benign mammary tumours developed in five out of eight rats. Most of the mammary tumours were observed in the abdominal-inguinal mammary gland than cervical-thoracic mammary gland. The presence of the tumour was confirmed by physical examination and histopathology. We conducted zymography for detecting the activity of matrix metalloproteinases (MMPs) in both malignant and benign mammary tumour and also normal organs like heart, kidney, liver, spleen and blood from tumour bearing rats. The results indicated that, the activities of MMPs were similar in malignant and benign tumours. MMPs activities were higher in tumour tissues than non tumour tissues like blood, liver and spleen of MNU-treated rats. In heart and kidney no MMPs activities were observed. MNU injection resulted in mammary tumours with good reproducibility as witnessed by gross and histopathological observation. Further, the MMP activities were higher in tumour tissues which may help in tumour development and progression.

Key words: Mammary tumour; N-methyl-N-Nitrosourea (MNU); Matrix metalloproteinases (MMPs); Histopathology; Gelatin zymography.

Cancer is one of the leading causes of death worldwide affecting both human and animals, with 8 lakh new cases of cancer diagnosed in humans every year in India alone[1]. Mammary tumours are most frequently encountered in dogs, cats and women[2]. Mammary tumours in female dogs represent upto 70% of all neoplasms[3] and more than a quarter of unspayed female dogs will develop a mammary tumour during their lifetime. The risk is much lower for spayed female dogs. In female dogs, usually 50% of mammary tumours are benign and 50% are malignant however, few of the malignant mammary tumours are fatal[4,5].

Cancer associated proteases (CAPs) are a set of proteases that are usually absent or present at very low concentrations in normal tissues but are often highly up-regulated in cancerous tissues[6]. There is positive correlation between the

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severity of tumour and the secretion of various proteases[7]. Some of extensively studied CAPs include urokinase plasminogen activator (uPA), matrix metalloproteases (MMPs) and some of the cathepsins[8]. MMPs are probably the most studied CAPs; they are a family of proteolytic enzymes which play a major role in tumour invasion[9] and metastasis[10]. MMPs are mainly produced by host stromal cells in most carcinomas[9] and also many tumour cells themselves can express MMPs and are regarded as major molecules assisting tumour cells during metastasis[11]. It has recently gained attention for targeting MMPs as a new method of tumour-responsive drug delivery[12,13,14].

Mammary gland tumours in rodent models are extensively used for the study of mammary cancer of women because of their similarity in terms of tumour histology and hormone dependence[2,15]. Most of the tumour susceptibility research has been done on laboratory rats. The Wistar rats represent the best experimental model for the study oncogenesis and carcinogenesis pathways in different tumour types[16].

N-methyl-N-nitrosourea (MNU) is a direct-acting alkylating agent that interacts with DNA. Accumulation of mutations may enhance risk of cancer in target organs. MNU-induced carcinogenesis model is the most widely used method for the induction of mammary tumour in rats[17].

In the present study we sought to investigate the induction of mammary tumour in female Wistar rats using MNU, a chemical carcinogen and evaluation of MMP activities and histopathological changes.

MATERIALS AND METHODS

Female Wistar rats (n=8) of about 28-35 days of age were procured from Laboratory Animal Resources, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar. These were housed in appropriate cages in the experimental animal house of Molecular Biology Lab, Division of Veterinary Biotechnology and acclimatized for a period of about 12-15 days. The rats were provided *ad libitum* food and water, housed in air conditioned room with controlled temperature, humidity with an artificial light-dark cycle of 12:12 hours. The experimentation was carried out as per the guidelines and approval of Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Induction of mammary tumour and histopathology

MNU (MW 103.08, Sigma Aldrich) was used as a chemical carcinogen for induction of mammary tumour. After acclimatization, the female Wistar rats were administered with five doses of chemical carcinogen MNU @ 50 mg/kg body weight at weekly intervals for induction of mammary tumour. MNU was dissolved in normal saline solution (pH 4-5 adjusted by glacial acetic acid). The solution was injected intraperitoneally in the ventral midline of the animal (half way between the third and fourth pair of the mammary glands) following all necessary safety and sterile precautions[18,19]. Body weight of all the rats used in tumour induction experiment was measured at weekly interval. Animals were observed daily for clinical sign and symptoms and palpated twice a week for detection of any growth in the mammary glands. The visible tumour nodules were measured using Vernier calipers at 3 days interval after day of first detection. Tumour Samples harvested for histopathology exam were fixed into 10% buffered formalin and processed by paraffin technique and sections were stained with hematoxylin-eosin. For MMPs characterization, samples were snap freezed in liquid nitrogen and stored in -80°C.

MMPs activity by gelatin zymography

MMPs are a family of proteolytic enzymes which play a major role in angiogenesis, tumour invasion[9] and metastasis as evident from their over expression in many forms of human. In this study, MMP activity was studied using gelatin zymography as described by Chew *et al.*[20] with minor laboratory modifications. For this, stored frozen samples of tumour, heart, kidney, liver and spleen tissue lysates from mammary tumour bearing rats were thawed on ice and mixed with equal volume of sample buffer and were subjected to SDS-poly acrylamide gel co-polymerized with 0.1% w/v gelatin. The separating gel of 12% acrylamide (pH 8.8) was prepared using the gel casting assembly spacers of 1.5 mm thickness. Stacking gel (4.5% in Tris buffer of pH 6.8) was poured over it and comb was placed. The gel after polymerization was transferred into an electrophoretic apparatus and buffer chambers

were filled with gel running buffer. Each sample (30 µl) was loaded into different wells of the gel along with fresh blood sample from mammary tumour bearing rat. Positive control with predetermined MMPs activity and prestained molecular weight marker (PageRuler) were also loaded in separate well. The electrophoresis was carried out at 150 V till the dye reached the bottom of the separating gel. Gels were washed with 2.5% Triton X-100 for 1 h at room temperature (with three changes of solution) to remove SDS. Gels were then incubated for 24 h at 37°C in incubation buffer (50 mMTris-HCl, 150 mMNaCl, 5 mM $CaCl_2$, and 0.05% NaN_3). After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue (G-250; Sigma) in a mixture of methanol-acetic acid-water (2.5:1:6.5 v/v). The destaining was carried using aqueous 4% methanol and 8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark blue background.

RESULTS AND DISCUSSION

Rodent models are useful for understanding the initiation, promotion, and progression steps of mammary carcinogenesis[15]. The results of the present study indicated that intraperitoneal administration of MNU provides an extremely simple technique for inducing mammary tumours with good reproducibility and good percentage of mammary tumour induction by MNU in Wistar rats (62.5%) suggesting the utility of the model in the study of chemically-induced mammary carcinogenesis. The tumour in Wistar rats is one of the best experimental models to study oncogenesis and carcinogenesis pathways in different tumour types[16]. There are many ways to induce mammary tumours, but previous reports indicate MNU as a preferable carcinogen[21].

In present study, all animals were sacrificed by cervical dislocation. The mammary glands were evaluated for the presence of grossly detectable mammary tumours and the dissected animals with tumours were photographed (Fig.1). A number of alterations were observed in the morphology of mammary tissues in the rats injected with MNU. All tumours observed at necropsy were encapsulated and of solid consistency. Tumour was further confirmed by histopathological examination, which revealed proliferating neoplastic epithelial cells after H&E staining. Neoplastic cells showed various degrees of neoplasia even in the same tumour. Epithelial cells were enlarged with increased nucleus/cytoplasm ratio along with enlarged nucleoli. Histological examination showed epithelial differentiation, typical epithelial hyperplasia was observed and ducts containing more than three layers of epithelial cells, adenocarcinomas with many morphological types. The most common type observed was adenoid cystic carcinoma represented as layers of tumour cells with cystic spaces separating them (Fig.2).

MMPs activity by gelatin zymography

Matrix-metalloproteinases (MMPs) are synthesized as proenzymes and typically activated by proteolytic removal of a propeptide[22]. MMPs

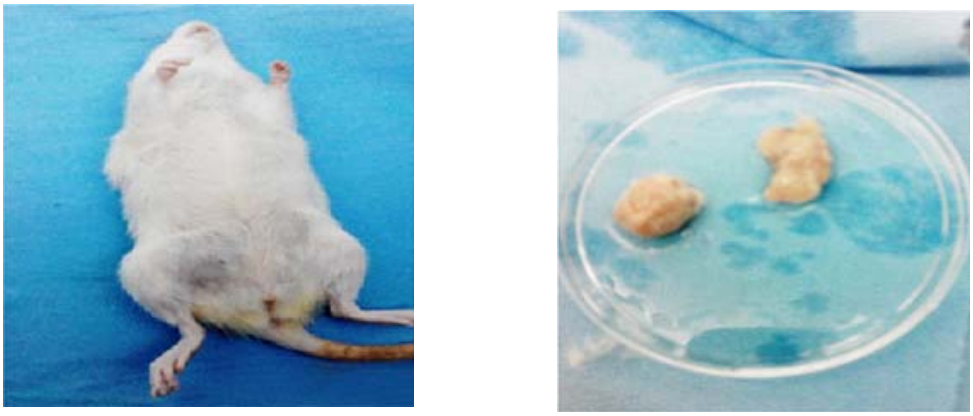

Fig. 1. A. Representative photograph of mammary tumour developed at last pair of mammary gland induced by MNU (A). Post operative mammary tumour tissues (B).

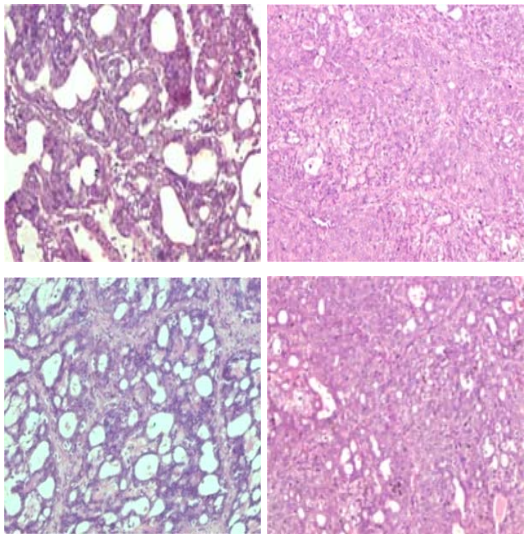

Fig. 2. Representative photomicrographs of mammary tumour sections, induced by MNU showing proliferating neoplastic epithelial cells after H&E staining.

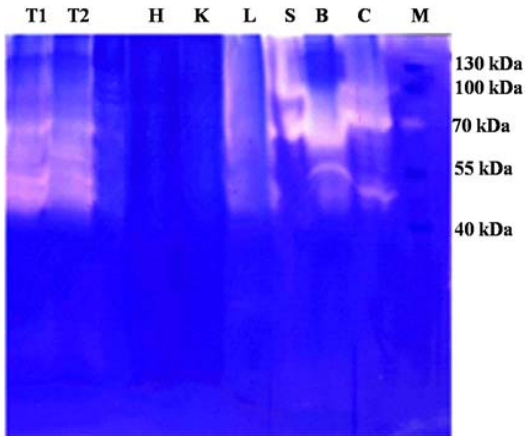

Fig. 3. Gelatin zymography showing presence of MMPs activity in Benign (T1), Malignant (T2) mammary tumour, Liver (L), Spleen (S) and Blood (B). MMPs activity was absent in Heart (H) and Kidney (K) of mammary tumour bearing rat. Positive control containing MMPs (C) and Prestained molecular weight marker-PageRuler (M).

are reported to influence tumour progression by facilitating events pivotal for neovascularization and establishment of distant metastasis including proliferation, survival and migration of endothelial, tumour and stromal cells[23,24]. MMP-2 and MMP-9 are implicated as prerequisites for angiogenesis and metastasis in the carcinogenic process. MMP-2 is expressed in the various cancer cell lines[25]. In contrast, MMP-9 has very limited or no expression in these cancer cells. Instead, MMP-9 is well-known to be secreted from cancer stromal fibroblasts and endothelial cells are a prerequisite for cancer angiogenesis[26,27]. In present study, gelatinase activities of the MMPs were identified by the gelatin zymography. Frozen samples of rat mammary tumours (both benign and metastatic), heart, kidney, liver and spleen and fresh blood sample from tumour bearing rat were used for analysis of gelatinolytic MMPs along with and positive control sample. Result indicated the activity of MMP-2 and MMP-9 was higher in both benign and malignant rat tumours compared to normal tissues However; the area was similarly extended in both benign and malignant rat tumours indicating there were no differences of MMPs activities between benign and malignant rat tumours. Similar observation was also reported by Taguchi *et al*[28]. These results indicated that the mammary tumours resulted may be due to high level of MMPs activities. Further our study also demonstrated the presence of MMPs activity in blood. Liver showed slight diffused type of MMP activity whereas, spleen showed only MMP9 activity but not MMP2. The remaining organs like Heart and kidney samples did not showed any MMPs activity (Fig. 3).

In conclusion, the results obtained shows that the MNU-induced mammary tumour model in Wistar rats can be developed with good reproducibiliy. The induced tumours showed discrete gross and histopathological changes with significant MMP activities in neoplastic tissues.

ACKNOWLEDGEMENTS

The authors are thankful to the authorities of Indian Veterinary Research Institute, Izatnagar, for granting permission to use rats for experiments. This work carried out was funded by NAIP (project code C4/C3001).

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