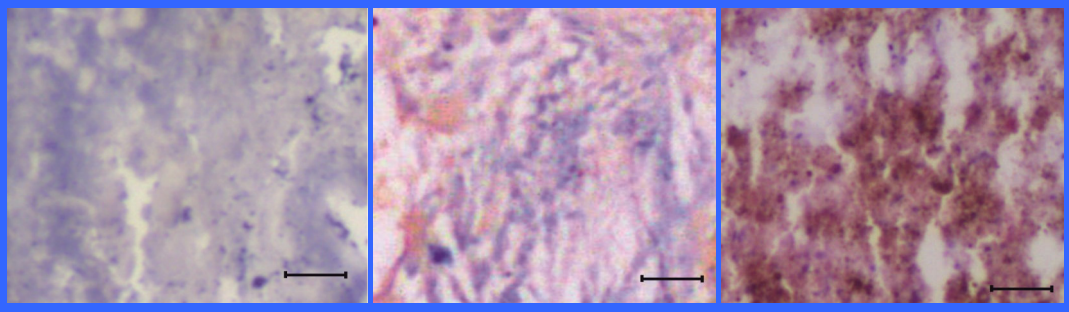

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Potential role of P2X7 receptor in regulating kidney stem cells in the course of acute kidney injury

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Abstract

The role of the P2X7R in developing acute kidney injury is unknown. In this study, we developed acute kidney injury mouse model system using 0.75% adenine and examined for renal damage using histology on day 2 and day 4. P2X7 antagonist (A438079) was used to study the recovery process after initial damage and it shows positive histological data. The P2X7R expression on the day 2 and day 4 of acute kidney injury was studied using immunohistochemistry and Western blotting. Result shows elevated expression as acute kidney injury progress. Later the P2X7R expression was compared with apoptotic signal and stem cell specific marker (CD133). The results conclude that the apoptotic signals are mainly associated with advanced stage of acute kidney injury but not much in day 2. Similarly, CD133 expression was masked in latter stages of injury following elevated expression in the initial stages.

Introduction

Acute kidney injury is the form of kidney injury that develops in an immediate manner (Mehta et al., 2007) after a particular causes like low blood pressure, on exposure to destructive substances, following inflammation in kidney or may be due to improper flow of urine. The patient with acute kidney injury faces 30-40% mortality (Lameire et al., 2006) even it has sub lethal damage of kidney cells and their mortality rates increase further in chronic kidney damage as it is suffering with lethal damages (Coca et al., 2009).

The assessing the kidney damage together with their functional aspect are poorly understood (Murugan and Kellum, 2011). Even after a lot of research there is no specific improvement in this field with suitable therapy to treat acute kidney injury other than some development in supportive care (Murugan and Kellum, 2011). The problem with acute kidney injury is associated with cell death that are mediated by either apoptosis or through necrosis (Bonventre and Yang, 2011).

P2X7 is a membrane receptors that are mainly associated with the large pore formation on activation by ATP that results to form membrane blabbing to initiate cell death (Birch et al., 2013). Studies with P2X7R expression demonstrate that its expression in normal kidney is under, low level (Turner et al., 2002) but in different pathological condition their expression elevated to the marked level (Harada et al., 2000). Still the basic functions of P2X7 in different pathological condition of acute kidney injury are largely unexplored. In this paper, we are assessing the role of the P2X7R in different condition of acute kidney injury together to find out their correlation they have with kidney stem cells in their recovery process.

Materials and Methods

Experimental animal

The healthy male mice of strain (C57BL/6) were used (age: 8 weeks; weight: 15-20 g). All the mice were kept

in a well ventilated room. The mice were developed with acute kidney injury by ingestion of 0.75% adenine (Morishita et al., 2011). The life of the mice ingested with 0.75% adenine cast can tolerate up to a maximum of 6 days. Following ingestion, the mice were analyzed for kidney damage on day 2 and day 4. One group of mice was injected with P2X7 antagonist (A438079) as previously described (Taylor et al., 2009) on day 2 of post-ingestion of 0.75% adenine and their recovery from developing acute kidney injury was studied on day 4.

Immunohistochemistry

For performing immunohistochemistry, the tissues were initially fixed with formalin and embedded in paraffin (Ascon et al., 2006). They were then subjected to thin sectioning of size 6 µm. The sections were then de-paraffinized using two times of xylene wash and dehydrated with isopropyl alcohol. The steps were followed with antigen retrieval using Tris EDTA (pH 9.0) and after washing they were immersed in 10% H_2O_2 solution for 30 min to mask the endogenous peroxidase. The sections were then incubated with 4% BSA solution for 30 min to minimize non-specific binding and latter the sections incubate with suitable antigen specific primary antibody, Anti-P2RX7 antibody (ab77413, abcam) or with Anti-CD133 antibody (ab19898, abcam) for 8 hours at 4°C. After washing the sections thoroughly with 1x PBS, they were incubated with suitable secondary antibody for 1 hour at room temperature. After washing for two times the sections were specifically bound to suitable secondary antibody. After throughout washing the specifically bind antibody are further stained using diaminobenzidine kit.

TUNEL assay

Apoptotic cells show a characteristic DNA fragment which were identified using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Fulda and Pervaiz, 2010). End labelled of DNA fragments was carried out by incorporating Brdu, a thymidine analogue to the 3′ ends of DNA strands. The incorporated Brdu in the apoptotic cell were then distinguished using anti-Brdu antibody (EMD Millipore, 05-633) following staining with diaminobenzidine kit.

Western blotting

The protein lysate was prepared from normal mice kidney and from the mice developed with acute kidney injury. For acute kidney injury developing mice, the samples were collected after day 2 and day 4 after 0.75% adenine injection. The protein samples were initially resolved in 12% SDS-PAGE gel and the further protocol was followed according with the previously described procedure (Hamidouche et al., 2008). For performing Western blotting, the membranes were initially primed with either anti-P2RX7 antibody (ab77413, abcam), anti-CAD antibody (C7852, Sigma-Aldrich) or with Anti-CD133 antibody (ab19898, abcam) and their signals were visualized with the help of suitable secondary antibody together with signalling reagents.

Results

Assessing the damage following adenine ingestion

Following 0.75% adenine we observed significant histological changes (Figure 1). The control kidney shows normal structures with compact tissue pattern. But after intake of mice with 0.75% adenine the kidney shows pathological changes and in particularly the compact nature of tissue pattern was disturbed. On the 2nd day of post-exposure with 0.75% adenine, the dense packing of solid tissue shows initial damages with widen renal tissue, together with enlarged nucleus. The condition proves worsen on day 4 with unpacked loosen tissue structure of the affected kidney.

Rescue with P2X7 antagonist

A438079 a chemical that helps to specifically target P2X7R and aids in potentially block them. The P2X7R is thought to be involved in initial pathogenesis of acute kidney injury. The mice developed with initial form of AKI were treated with P2X7 antagonist (100 mg/kg, ip) on the day 2. Once the P2X7 antagonist absorbed in the body system, it exerted a protective role. On examining the histological kidney tissue on the day 4, we observed significant restoration of abnormal tissue pattern as that of normal tissue (Figure 1D). The affected tissue pattern reverted and it was observed with a progressive histological pattern that is similar to that of control.

Correlation of P2X7R expression with apoptotic and CD133 expression

In the present study, the obtained histological data were further analyzed in depth using specific molecular markers that helps to understand whether P2X7R has a role of apoptosis or growth stimulating role. The normal kidney tissue had limited expression of P2X7R (Figure 2A) but by inducing acute kidney injury their expression got up-regulated (Figure 2B, C). The more specific observation was P2X7R expression enhanced with severe damage to kidney tissue as observed on day 4 of acute kidney injury (Figure 2C).

The role of P2X7R expression in the developing pattern of AKI was further studied using an apoptotic assay (Figure 2D-F). Surprisingly, we observed the apoptotic signal that shows on day 2 of acute kidney injury (Figure 2E) was magnified as the acute kidney injury progress that is on the day 4 (Figure 2F). The results concluded the acute kidney injury developed on day 4 showed severe cell death and at this stage the acute kidney injury could not be reverted. Similarly the corre-

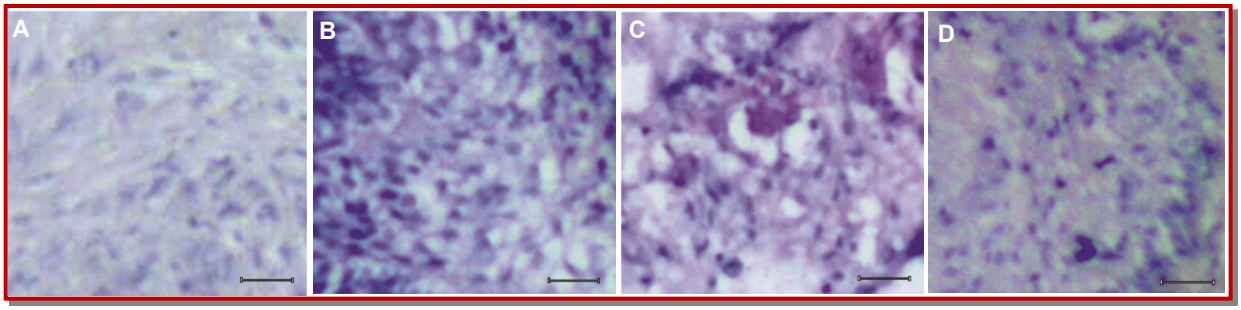

Figure 1: Transverse section of mice kidney. Normal mice kidney showing compact arrangement of cells (A). Moderate damage of mice kidney following 0.75% adenine ingestion on day 2 (B). Histology of mice kidney developed acute kidney injury on day 4, which shows more disturbed tissue pattern (C). Rescue with P2X7 antagonist (D)

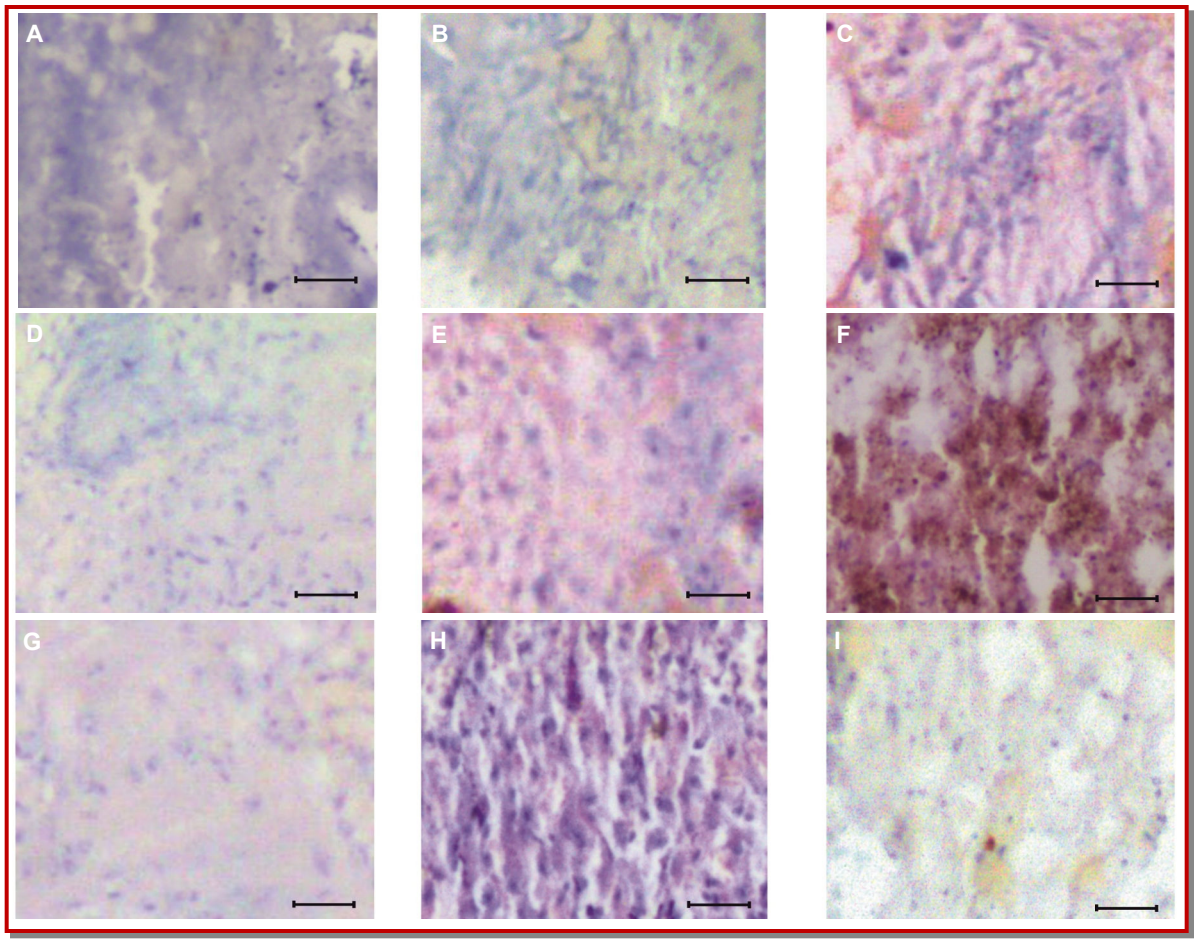

Figure 2: Comparative analysis of P2X7R, apoptotic and CD133 expression in normal and acute kidney injury tissue. Normal mice kidney shows P2X7R expression (A). P2X7R expression starts to elevate on day 2 of acute kidney injury (B). High expression of P2X7R in advance stage of acute kidney injury (C). Control tissue without apoptotic signal (D). Moderate apoptotic signals on day 2 of acute kidney injury (E). Highly elevated expression of apoptotic protein on day 2 of acute kidney injury (F). Expression of renal stem cell specific protein in normal tissue (G). Elevated CD133 expression in initial damage of acute kidney injury on day 2 (H). Significantly down-regulated expression of CD133 in severe damage condition of acute kidney injury on day 4 (I). Scale Bar = 100 µm size

lation between P2X7 expression and CD133 expression were analyzed (Figure 2G-I). We observed CD133 was barely expressed in control tissue (Figure 2G) and it shows elevated expression on day 2 of acute kidney injury (Figure 2H). But its specific pattern of expression gets diluted on day 4 of acute kidney injury (Figure 2I).

The specific signals obtained through immunohistochemistry were further analysed using Western blotting technique. The results confirms that P2X7R over express as acute kidney injury progress (Figure 3, Lane 1-3) and similarly apoptotic activity shows elevated expression on day 4 (Figure 3, Lane 6) of

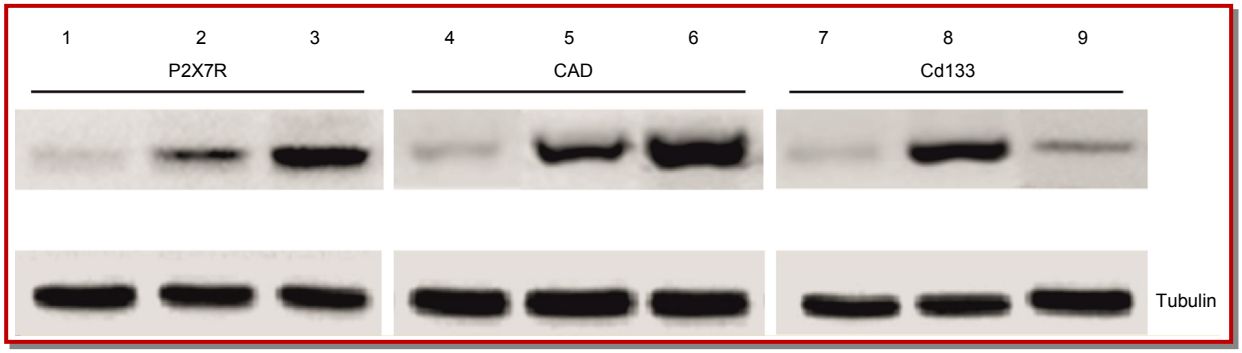

Figure 3: Expression pattern of P2X7R, apoptotic and CD133 in normal and acute kidney injury tissue. Lane 1 to lane 3 represents P2X7R expression in normal kidney tissue, samples from acute kidney injury tissues of day 2 and day 4 respectively. Lane 4 to lane 6 represents CAD expression in normal kidney tissue, samples from acute kidney injury tissues of day 2 and day 4 respectively. Lane 6 to lane 9 represents CD133 expression in normal kidney tissue, samples from acute kidney injury tissues of day 2 and day 4 respectively. For all the experiments, tubulin was used as the loading control

acute kidney injury when compare it with day 2 (Figure 3, Lane 5). But the expression of CD133 was rapidly down-regulated on day 4 (Figure 3, Lane 9) following over expression as observed on day 2 (Figure 3, Lane 8). For the control purpose tubulin was used.

Discussion

Normally the kidney express a very low level of P2X7R (Turner et al., 2002) as our results suggest but its elevated expression are observed in many abnormal conditions like chronic kidney disease (Ji et al., 2012). Recently the therapy based on P2X7R in cirrhosis condition are revealed out (Adebayo et al., 2013). Adenine which stimulates acute kidney injury formation was validated with histological examination. Our histological studies confirm as the acute kidney injury progress the condition of renal damage significantly worsen and with P2X7 antagonist (A438079) the damage can be reverted to certain extend. The results suggest the P2X7 antagonist (A438079) interact in an effective manner and further studies in this direction give more defined understanding in this area of research.

The role of the P2X7R in cell growth and apoptosis remains unclear. Here we pointed out that it has triggered apoptosis signal in advanced acute kidney injury when comparing with initial day 2 acute kidney injury. The results give a conclusion that P2X7R is important for regulation of advance stage of acute kidney injury. Other than this our results demonstrate the role of the P2X7R in triggering stem cells in initial damage but when the damage become severe the stem cell population gradually reduced which confirms the unable situation to revert the damage. All the results obtained through immunohistochemistry are further analysed and confirmed using western blotting. The comparative analysis of both the data shows almost similar results which helps to validate the obtained data.

Conclusion

P2X7 assists in forming acute kidney injury. The progress in acute kidney injury is associated with increasing in expression of P2X7R. Other than that the specific investigation with apoptotic and CD133 suggests the severe form of AKI supports elevated expression of apoptotic protein together with reduced expression of CD133. The comparative analysis reveals the regulative function of P2X7.

Ethical Issue

All the procedures to be followed for performing the experiments were approved by the institutional animal welfare and ethical committee.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgement

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