Effect of MLN8237, a Novel Aurora A Kinase Inhibitor, on the Spontaneous Fragmentation of Ovulated Mouse Oocytes

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ABSTRACT

Aurora A kinase is a mitotic serine/threonine kinase whose proposed functions include the maturation of centrosomes, G2/M transition, alignment of chromosomes at metaphase, and cytokinesis. In this study, we investigated the effect of MLN8237, an aurora A kinase inhibitor, on the postovulatory aging of oocytes based on the frequency of oocyte fragmentation, cdk1 kinase activity, and cyclin B degradation. The fragmentation of ovulated oocytes during prolonged culture was inhibited by treatment with MLN8237 in a concentration-dependent manner. The frequency of fragmented oocytes was significantly lower in oocytes treated with 2 μM MLN8237 (13%) than in control oocytes (64%) after two days of culture. Most of the control (non-fragmented) oocytes (91%) were activated after two days of culture. In comparison, only 22% of the MLN8237-treated oocytes were activated; the rest of the oocytes (78%) were still in metaphase with an abnormal spindle and dispersed chromosomes. Next, cdk1 activity and the level of cyclin B were examined. The level of cyclin B and cdk1 activity in MLN8237-treated oocytes were nearly equal to those in control oocytes. Our results indicate that MLN8237 inhibited the fragmentation of ovulated oocytes during prolonged culture, although it blocked the spontaneous decrease in activity of cdk1 and degradation of cyclin B. This mechanism of inhibition is different from that in oocytes treated with nocodazole, which have high levels of cdk1 activity and cyclin B.

(Key words : Oocyte fragmentation, Aurora A kinase, MLN8237, cdk1 activity)

INTRODUCTION

In most mammalian species, ovulated oocytes are arrested at metaphase II and exhibit high levels of cdk1 activity; then, they re-enter interphase with extrusion of the second polar body at fertilization (Masui, 2000). It has been shown that one of the causes of postovulatory oocyte aging is a gradual decrease in cdk1 activity (Taton *et al.*, 2006; Igarashi *et al.*, 2005; Kikuchi *et al.*, 2000, 2002; Choi, 2011). Cdk1 kinase activity in ovulated oocytes is maintained via an activating spindle assembly checkpoint (SAC) by treatment with nocodazole, a spindle depolymerizing drug. In oocytes treated with nocodazole, spontaneous fragmentation, which is representative of postovulatory oocyte aging, is inhibited (Kubiak *et al.*, 1993).

Aurora kinases are serine/threonine kinases that play a critical role in cell cycle regulation (Crane *et al.*, 2003). In vertebrates, the aurora kinase family is composed of three members: aurora A, B, and C (Carmena and Earnshaw, 2003). Among these, aurora A kinase has been implicated in centrosome maturation, the G2/M transition, chromosome alignment at metaphase, and cytokinesis (Hoar *et al.*, 2007; Marumoto *et al.*, 2002). In proliferating cells, the inhibition of aurora A kinase induces a G2/M arrest, apoptosis, or senescence (Huck *et al.*, 2010; Gorgun *et al.*, 2011).

A previous study of mouse oocytes showed that aurora A kinase was expressed from germinal stage oocytes to ovulated oocytes, and that it was essential for both germinal vesicle breakdown (GVBD) and spindle formation (Yao *et al.*, 2004; Saskova *et al.*, 2008). However, there is no report on the functions of aurora A kinase in metaphase II-arrested oocytes. In this study, we investigated the functions of aurora A kinase in maintaining chromosome alignment and the spindle in metaphase II-arrested oocytes, as well as the effects of postovulatory aging using MLN8237, a novel inhibitor of aurora A kinase (Tomita and Mori, 2010).

MATERIALS AND METHODS

Oocyte Collection and Culture

Five- and six-week-old ICR female mice were used. To obtain ovulated oocytes, the mice were injected intraperitoneally with 5 IU of pregnant mare serum gona-

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dotropin (Sigma, St. Louis, MO, USA) and with 5 IU of human chorionic gonadotrophin (hCG; Sigma) 48 h later. Ovulated oocytes were collected from the ampullae of the oviducts 15~16 h after hCG injection. The oocytes were separated from the cumulus cells by incubation for 2~3 min in 0.1% hyaluronidase (Sigma) in M16 culture medium (Sigma) at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. The cumulus cell-free oocytes were rinsed three times. Only phenotypically normal oocytes were collected. MLN8237, an aurora A kinase inhibitor, was obtained from Selleck Chemicals (Houston, TX, USA). Nocodazole was purchased from Sigma. Both chemicals were dissolved in DMSO. The final concentration of DMSO in all experiments was ≤0.2%.

Evaluation of the Oocytes

Fragmented oocytes were observed by microscopy at the end of the culture period. We found two cell-like oocytes, which divided symmetrically from the fragmented oocytes. To investigate the spindle and chromosomes, the oocytes were fixed with 1.8% paraformaldehyde in PBS for 40 min and then permeabilized with 1% Triton X-100 in PBS for 20 min. The oocytes were then washed with 0.1% Tween 20 in PBS for 20 min, after which they were blocked with PBS containing 3% BSA (blocking solution) for 1 h at room temperature (RT). The oocytes were then incubated with anti-tubulin antibodies (YL1/2; Accurate Chemical) at a 1:100 dilution and then with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma) at a 1:50 dilution. For both the primary and secondary antibodies, incubation was done for 1 h at RT. The oocytes were co-stained with 4,6-diamidinophenylindole (DAPI) to visualize the DNA (Sigma). Immunostained oocytes were examined using an Olympus IX71 fluorescence microscope.

Measurement of cdk1

A cdk1 kinase assay was conducted according to the method described by Ito *et al.* (2005), with modifications. Briefly, 40 denuded oocytes were lysed in 5 μl of ice-cold RIPA buffer (including protease and phosphatase inhibitors; Sigma) and then frozen at −70℃ until use. Kinase activity in each oocyte lysate was measured using a MESACUP cdc2 kinase assay kit (MBL, Nagoya, Japan). All data are expressed as the relative percentage of the level of p34cdc2 kinase activity (ovulated oocytes defined as 100% and activated oocytes exposed to ethanol for 6 h as 0%).

Immunoblot Analysis of cyclin B1

A total of 50 oocytes in each group were used for immunoblotting. The oocytes were suspended in 10 μl of 2× Laemmli sample buffer, boiled for 5 min, and then subjected to standard 10% SDS-PAGE. The separated proteins were transferred to PVDF membranes and analyzed using anti-cyclin B antibodies (Millipore).

Statistical Analysis

Each experiment was replicated at least three times. Statistical differences between the groups were assessed by one-way ANOVA. The data are shown as the mean ±SEM; significant differences were defined as $p<0.05$.

RESULTS

Effect of MLN8237 on Oocyte Fragmentation

Ovulated oocytes cultured with or without MLN8237 were investigated on days 1 and 2 (Fig. 1). In control media, the frequency of oocyte fragmentation was 15% on day 1 and 64% on day 2. However, in MLN8237 media, a concentration-dependent decrease up to 2 μM was observed. In the case of nocodazole-treated oocytes, fragmentation was effectively inhibited by 2 μM MLN8237 (3 vs. 13%). After two days of culture, 91% (48/53) of the single (non-fragmented) oocytes were activated, while 22% (16/73) of the 2 μM MLN8237-treated oocytes were activated. The rest of the oocytes (78%) had abnormal or weak spindles and dispersed, condensed chromosomes (Fig. 2).

Levels of cyclin B and cdk1 Activity in MLN-treated Oocytes

We assessed whether oocytes cultured with MLN-8237 displayed cdk1 activity as described in the Materials and Methods. As shown in Fig. 3, kinase activity

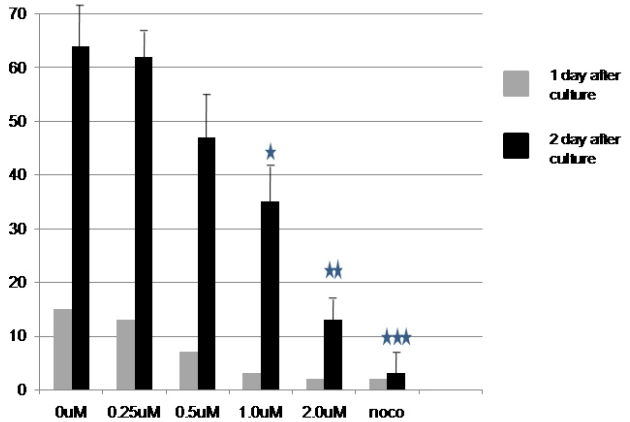

Fig. 1 The frequency of fragmented oocytes among single oocytes cultured with or without MLN8237 was evaluated on a daily basis. Spontaneous oocyte fragmentation was inhibited in a concentration-dependent manner by the addition of MLM8237 to the medium. The data represent the mean±SEM (n=3); values with different superscripts are significantly different ($p<0.05$).

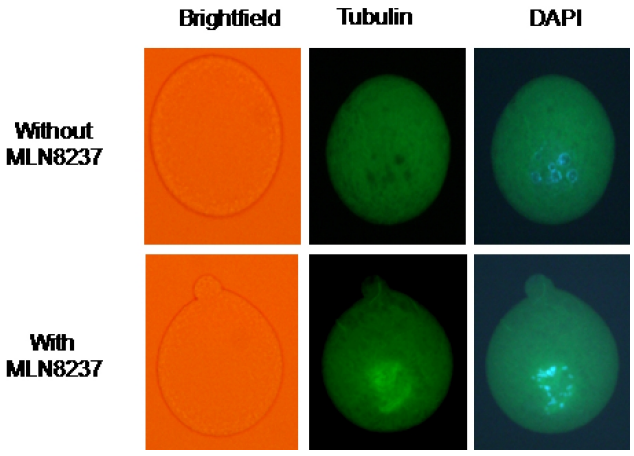

Fig. 2 Fluorescence photomicrographs of oocytes stained with anti-tubulin antibodies and DAPI. Oocytes with or without MLN8237 after two days culture were fixed and stained with anti-tubulin antibodies and DAPI. The oocytes cultured without MLN8237 possessed nuclei but no spindle; in comparison, the oocytes cultured with MLN8237 possessed abnormal spindles and scattered chromosomes.

in those oocytes cultured in control medium for one and two days showed a 92 and 27% decrease in cdk1 activity, respectively. The kinase activity of oocytes cultured with 2 μM MLN8237 was 98 and 35%, respectively; in comparison, in nocodazole-treated oocytes the level of kinase activity was 98 and 85%. It is well known that the inactivation of cdk1 during the transition from meiosis to interphase results from the degradation of cyclin B. Therefore, we measured the cyclin B protein levels in oocytes cultured with or without MLN

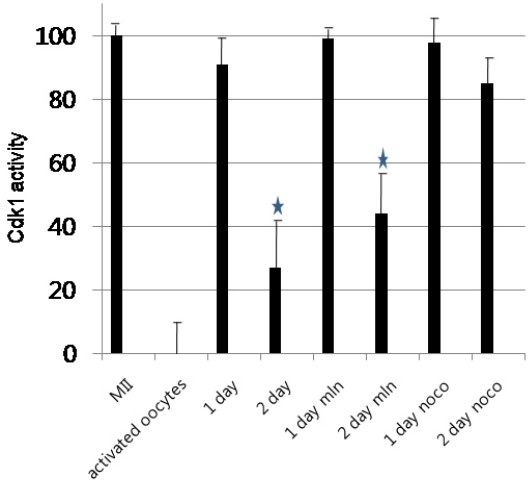

Fig. 3 Kinetics of cdk1 activity in oocytes during *in vitro* culture with or without MLN8237. Cdk1 activity was calculated as a percentage of the maximal level of activity (the level of activity in ovulated oocytes was defined as 100%, while that in activated oocytes exposed to ethanol for 6 h was defined as 0%). The decrease in cdk1 activity caused by prolonged culture in the ovulated oocytes was delayed by the addition of MLN8237 to the culture medium.

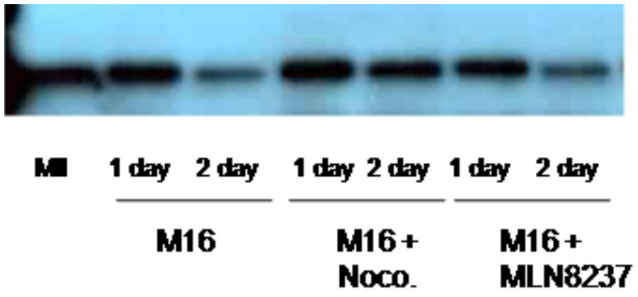

Fig. 4 Changes in the level of cyclin B in oocytes cultured for two days with or without MLN8237. Lysates prepared from 50 oocytes were analyzed by immunoblotting using anti-cyclin B antibodies.

8237 or nocodazole by immunoblotting. As shown in Fig. 4, MLN8237 treatment did not inhibit cyclin B degradation; however, nocodazole treatment inhibited the degradation of cyclin B. Our results show that cdk1 activity is correlated with the degradation of cyclin B.

DISCUSSION

Aurora A kinase has been implicated in centrosome maturation, the G2/M transition, chromosome alignment at metaphase, and cytokinesis (Vader and Lens, 2008)). In proliferating cells, the inhibition of aurora A kinase induces a G2/M arrest, apoptosis, or senescence (Hoar *et al.*, 2007; Marumoto *et al.*, 2002; Huck *et al.*, 2010; Gorgun *et al.*, 2011) in different cell lines and under different culture conditions. A previous study of mouse oocytes revealed that aurora A kinase was required for GVBD and proper spindle formation in meiosis I and II (Yao *et al.*, 2004; Saskova *et al.*, 2008). Although many of the functions of aurora A kinase during oocyte maturation have been studied, its functions in ovulated oocytes, which are arrested at metaphase II, have not been reported.

In the present study, we investigated the function of aurora A kinase in metaphase II-arrested oocytes, and its effect on postovulatory aging using MLN8237, a novel inhibitor of aurora A kinase. Our results showed that MLN8237 treatment induced disruption of the spindle and chromosome scattering, but that it inhibited spontaneous oocyte fragmentation and activation.

We expected that MLN8237 would induce the SAC and inhibition of cyclin B degradation while maintaining cdk1 activity, similar to nocodazole (Kubiak *et al.*, 1993). However, our results showed that MLN8237 did not induce the SAC. However, oocytes treated with MLN8237 for two days had an abnormal spindle and chromosomes, despite the decline in cdk1 activity.

We cannot explain the reason for the presence of the spindle and chromosomes in the present study; how-

ever, one explanation is that treatment with MLN8237 blocked the degradation of other proteins required for the exit from mitosis, except for cyclin B. A previous study showed that cultured cells maintained mitosis without cdk1 activity (Scofias *et al.*, 2007). In that paper, the authors reported that the degradation of cyclin A and emi 1 in addition to cyclin B and securin was required for the exit from mitosis.

Another possible explanation for our finding is that MLN8237 weakened microtubule polymerization. Microtubule staining in oocytes treated with MLN8237 produced an extremely blurry signal. Previous studies have shown that microtubules are required for cell fragmentation during apoptosis (Moss *et al.*, 2006).

In summary, the inhibition of aurora A kinase by MLN-8237 in ovulated oocytes decreased oocyte fragmentation and activation without affecting cdk1 activity.

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