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Streptozotocin, an *O*-GlcNAcase Inhibitor, Stimulates TNFα-Induced Cell Death

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Abstract

O-GlcNAcylation of p53 has been already identified and reported, but the function of *O*-GlcNAc on p53 has not been studied well. In this report, the general function of *O*-GlcNAc modification on p53 has been investigated using mouse fibroblast cell, L929. When streptozotocin (STZ), a non-competitive *O*-GlcNAcase inhibitor was treated to L929, *O*-GlcNAc modification level was dramatically increased on nucleocytoplasmic proteins, including p53. Because it has been already reported that TNFα induced the production of p53 in L929, TNFα was treated to obtain more p53. Approximately two times more amount of p53 was found from the cells treated STZ and TNFα simultaneously compared to the cell treated TNFα alone. The p53 increment in the presence of STZ was not caused by the induction of p53 gene expression. When new production of p53 induced by the TNFα was inhibited by the treatment of cycloheximide, *O*-GlcNAc modification decreased and phosphorylation increased on pre-existing p53 after TNFα treatment. But in the presence of STZ and TNFα at the same time, more O-GlcNAcylation occurred on p53. The level of ubiquitination on p53 was also reduced in the presence of STZ. Approximately three times less amount of Mdm2 bound to this hyperglycosylated p53. From this result it might be concluded that treatment of STZ to inhibit *O*-GlcNAcase increased *O*-GlcNAc modification level on p53 and the increment of *O*-GlcNAc modification stabilized p53 from ubiquitin proteolysis system.

Results and Discussion

Effects of Streptozotocin on p53 in L929 Cell Lines

Because *O*-GlcNAc modification on nucleocytoplasmic proteins is very dynamic, if STZ, a non-competitive inhibitor of *O*-GlcNAcase is treated to the cell, *O*-GlcNAcylated glycoproteins in nucleocytoplasm would be accumulated. In fact, L929 cells treated with 5 mM of STZ of which concentration did not affect L929 cell liability contained more *O*-GlcNAcylated glycoproteins compared to those not treated with STZ. Tumor suppressor p53 has been reported as an *O*-GlcNAcylated glycoprotein, and p53 of L929 was also modified with *O*-GlcNAc. Therefore, there will be a possibility that L929 cells treated with STZ can contain more p53 modified with *O*-GlcNAc than those not treated with STZ. There were two reports that more p53 proteins were

accumulated in L929 as a result of elevating p53 gene expression after treatment of TNFα to the cells. Therefore, if TNFα and STZ are treated to the cells at the same time, it would be expected more amount of p53 modified with more *O*-GlcNAc from the cells and it would be necessary to know the site of *O*-GlcNAc modification on p53 and its function. To prove this hypothesis, the amount of p53 present in L929 cells, which were pretreated with 5mM STZ for 8 hours and then treated with TNFα was investigated using Western blot. As expected, after 24 hours 2.5 times more amount of p53 was found from L929 cells only treated with TNFα. Interestingly, two times more amount of p53 was found in the cells treated with TNFα and STZ at the same time than those treated with only TNFα and this result was totally unexpected. There would be a possibility that STZ also induced gene expression of p53 like TNFα. To prove this possibility, the mRNA level of p53 was investigated at various conditions using RT-PCR. The mRNA level of p53 increased three times after 8 hour TNFα treatment but STZ did not affect the mRNA level of p53. Therefore, increment of p53 in the cells treated with STZ was not due to induction of p53 gene expression. From this result, it would be assumed that the change of modification on p53 by STZ was a key for the increment of p53.

Dynamic changes of phosphorylation and *O*-GlcNAcylation of p53

Various modifications are found on p53 such as phosphorylation, acetylation, *O*-GlcNAcylation, SUMOylation, ubiquitination, etc. Among these modifications, especially phosphorylation and *O*-GlcNAcylation share same modification sites, Ser and Thr. Therefore, there would be a reciprocal competition for the same sites between these two modifications or if sites are close to each other, one modification would influence the other. To know the change of modifications on pre-existing p53 under the treatment of TNFα, phosphorylation and *O*-GlcNAcylation on p53 were investigated using Western blot in the presence of cychloheximide to inhibit new production of p53. The phosphorylation on p53 was increased gradually by 12 hours and *O*-GlcNAcylation was decreased. This result means that both modifications on p53 in L929 may influence to each other. In order to investigate the change of modification on pre-existing p53 under various conditions, p53 was immunoprecipitated and analyzed using 2-DE and Western blot. The p53 was obtained from L929 treated STZ, TNFα or TNFα and STZ at the same time for 12 hours, respectively. The p53 from the cell treated with STZ was modified with *O*-GlcNAc more than that from the cell not treated. In the case that TNFα was treated only to the cells, more phosphates were attached to the various sites of p53 and amount of p53 modified with *O*-GlcNAc was reduced. Interestingly, in the case that STZ and TNF α were treated at the same time, more *O*-GlcNAcylation occurred on p53 instead of phosphorylation. Under the condition that *O*-GlcNAcase was inhibited by STZ treatment, hyperglycosylated p53 presented in the cell. Therefore, more amount of p53, probably hyperglycosylated p53, presented in the cell treated with TNFα and STZ at the same time than treated with TNFα only meant that more *O*-GlcNAcylation occurred on p53 might make it more stable.

Ubiquitination of p53

The p53 is a short-lived protein. The production or degradation of p53 is induced in response to

particular types of stress. The uniquitination of p53 is a major pathway to degrade itself. Therefore, ubiquitination of p53 obtained from STZ treated or not treated L929 was compared. The p53 from cells cultured as described above was immunoprecipitated at indicated time points and total ubiquitination of p53 was analyzed using ubiquitin antibody. The ubiquitination of p53 occurred less in the cell treated with STZ and TNFα simultaneously than in the cell treated with TNFα only at 12 and 24 hour incubation. It was assumed that less ubiquitination occurred on hyperglycosylated p53, which presented in the cell treated with STZ, and the hyperglycosylated p53 was not degraded via ubiquitin system. In result, the hyperglycosylated p53 might be accumulated in the cells treated with STZ.

Binding of hyperglycosylated p53 to Mdm2

Direct physical contact between p53 and Mdm2 inactivates p53 because Mdm2 performs as an E3 ligase to ubiquitinate p53 and propels its proteasomal degradation. Because less ubiquitination occurred on hyperglycosylated p53, binding of p53 to Mdm2 was investigated to know whether hyperglycosylated p53 binds less to Mdm2. The p53 from cells cultured in the presence or absence of STZ under TNFα treatment was immunoprecipitated at indicated time points and the amount of coimmunoprecipitated Mdm2 was analyzed using Mdm2 antibody. In result, hyperglycosylated p53 obtained from the cells treated with STZ bound much less to Mdm2. Conclusively, hyperglycosylated p53, which presented in STZ treated cells, binds less to Mdm2 and, in result, less ubiquitination occurred on hyperglycosylated p53. Therefore, *O*-GlcNAc modification on p53 gives its stability.

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