主論文の要旨

Non-thermal plasma induces a stress response in mesothelioma cells resulting in increased endocytosis, lysosome biogenesis and autophagy

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<Background>

Malignant mesothelioma is a rare, but highly aggressive cancer in humans that occurs after exposure to asbestos. An interesting new form of therapy to malignant mesothelioma involves the generation of non-thermal plasma (NTP). NTP is a partially ionized gas in which energy is stored predominantly as free electrons and the temperature remains as low as body temperature. For many years, NTP has been used in a variety of applications, such as low-temperature plasma chemistry and the removal of gaseous pollutants. Recent studies have demonstrated that NTP shows antitumor activity in vitro against a range of tumor cell-types, via the induction of DNA damage, cell cycle arrest and apoptosis.

While the cellular response to NTP in terms of ROS generation, cell cycle arrest and apoptosis has been characterized, the role of iron, ROS and autophagy in terms of the biological efficacy of NTP has not been assessed. This is important to evaluate, considering that iron can potentiate ROS generation, and thus, tumor cell killing. The current investigation demonstrates for the first time that NTP is selectively cytotoxic to mesothelioma cells and induces a cellular stress response probably mediated via iron-induced ROS generation. This response consists of a marked increase in fluid-phase endocytosis/pinocytosis, early endosome formation, lysosome biogenesis and autophagy. The pronounced induction of autophagy by NTP is important in terms of understanding the cellular mechanism of action of this new treatment modality.

<Materials and Methods>

Four cell lines were used in this experiment: the human fibroblast cell line IMR 90SV, rat fibroblast cell line Rat-1, the rat SM2 (sarcomatoid sub-type) and EM2 (epithelioid sub-type) malignant mesothelioma cells. Different assays were performed on the mesothelioma cells such as immunoblotting, gel shift assay, fluorescence staining and transmission electron microscopy.

<Rsults>

Our initial experiments examined the activity of NTP in malignant mesothelioma cells (SM2 and EM2) relative to fibroblast cell line (IMR 90SV; Fig. 1Aii). In contrast to cisplatin (Fig.1Ai), an appropriate reference chemotherapeutic, NTP demonstrated greater selective anti-proliferative activity against both malignant mesothelioma cell lines relative to the IMR 90SV fibroblast cell line. Notably, argon gas alone had no significant effect on cellular proliferation (Fig. 1B). Studies designed to assess the effects of cellular iron-loading and -depletion on the anti-proliferative efficacy of NTP using the SM2 mesothelioma cells. In fact,

preincubation with ferric ammonium citrate (FAC) resulted in a marked and significant increase in the anti-proliferative efficacy of NTP (Fig. 1Ci). Interestingly, incubation with desferrioxamine (DFO), a redox-inactive and clinically implemented "gold standard" iron chelator, at all concentrations tested markedly and significantly prevented the anti-proliferative activity of NTP (Fig. 1Cii).

To assess intracellular ROS generation, three well characterized redox-sensitive probes were utilized, namely, CMH2DCF-DA, MitoSOX and C11-BODIPY. Treatment with NTP resulted in a change in the cytometric profile, indicating ROS generation in the cells. This effect was markedly prevented by pre-incubation with the chelator, DFO (NTP+DFO), suggesting the role of iron in NTP-generated ROS (Fig. 2Ai-iii). For both the SM2 and EM2 malignant mesothelioma cell lines, the fold change in ROS generation after incubation with NTP relative to the control (CTRL) was significantly greater for each individual probe than that found for the fibroblasts (Fig. 2Bi–iii).

Our experiments then assessed the effect of NTP on the expression of proteins involved in iron metabolism. Relative to the 0 h time point, the expression of both IRP1 and IRP2 proteins was significantly decreased after NTP exposure times of 4–24 h and 0.5–24 h, respectively (Fig. 3A, B). Furthermore, a significant decrease in transferrin receptor 1 (TfR1) was observed from 2 to 24 h (Fig. 3C). Examining ferritin-H (Fig. 3D) and ferritin-L (Fig. 3E) expression, there was transient and significant increase between 4–18 h and 2–4 h, respectively.

As IRP-IRE-binding is reduced upon increased intracellular iron, studies assessed this using a well-characterized gel-shift assay. The binding of IRP1 to the IRE mRNA probe was slightly decreased following NTP and a 2-h incubation and this became a significant decrease in IRP1-IRE binding after a 4- and 8-h incubation after NTP treatment (Fig. 4A). Assay examining the co-localization of catalytic Fe(II) within lysosomes was performed, a marked and significant increase in the intensity of the punctate HMRhoNox-M and LysoTracker staining after NTP treatment (Fig. 4B).

Treatment of SM2 cells with NTP (60 s) followed by an incubation for 4 or 8 h caused a significant time-dependent increase in pHrodo Red Dextran uptake (red) after 4 h or 8 h. In contrast, EEA1 (green) staining increased significantly after 4 h and then decreased to near control levels at 8 h (Fig. 5A). Similarly, western analysis also suggested a slight, but significant increase in EEA1 expression after exposure to NTP at 4, 18 and 24 h of incubation (Fig. 5B). After treatment of SM2 cells with NTP (60 s) and an incubation of 4 h, there was a marked and significant increase in the staining of both lysosomal markers Pepstatin A Bodipy FL conjugate (green) and LysoTracker (red) resulting in yellow fluorescence (Fig. 5C). And the increasing of LAMP1 was confirmed again with western blot analysis (Fig. 5D).

To directly assess the induction of autophagy, LC3B, a classical marker of the autophagosome that directly correlates with autophagosome number was examined. NTP induced a pronounced and significant increase in LC3B (red) and LAMP1 (green) fluorescence intensity (Fig. 6A). And based on immunoblotting assays, the LAMP1 band was significantly increased after 4 and 8 h. On the other bands, the levels of LC3B-I expression were not significantly altered at all incubation times. In contrast, a marked and significant increase in the LC3B-II level relative to the control was evident after a 0.5-h incubation (Fig. 6B). Both LC3B-I and LC3B-II expression were markedly increased in NTP treated SM2 and EM2 mesothelioma cells while the change in fibroblast IMR 90SV was very slight (Fig. 6C). And treatment of NTP with FAC at non-toxic doses induced great increasing in LAMP1 (Green) and LC3B (Red) relative to with NTP or FAC treatment alone (Fig 6D).

We performed transmission electron microscopy to assess the presence of endosomes, lysosomes and autophagic vacuoles in SM2 cells after NTP. In CTRL SM2 cells, very few autophagic vacuoles were apparent (Fig. 7Ai–Di), however, upon treatment with NTP, there was a marked alteration in cellular morphology with a pronounced increase in autophagic vacuoles being obvious (Fig. 7Bii–Dii).

<Discussion>

For the first time, NTP was demonstrated to induce a stress response in malignant mesothelioma cells that constituted increased oxidative stress, endocytosis, lysosome formation and autophagy. This effect was important to characterize in terms of understanding the anti-proliferative activity and efficacy of NTP against neoplastic mesothelioma cells.

These studies indicate a mechanism by which NTP induces autophagy via ROS generation, increased fluid-phase endocytosis, an increase of early endosomes and lysosomes, and the induction of autophagic vesicles. Such a response to NTP involving autophagy could be induced as a protective repair mechanism that leads to the cellular turnover of proteins and organelles that include iron-containing constituents, leading to the liberation of the metal in lysosomes. A similar cellular response has been reported in cells incubated under other stress stimuli.

<Conclusion>

In summary, this investigation has demonstrated for the first time that NTP markedly increases cellular ROS levels with the generation of these species being dependent on intracellular iron. Hence, through this mechanism, NTP acts as a stress stimulus and results in an increase in endocytosis, early endosome and lysosome biogenesis, and the induction of autophagy. These results are important

for understanding the mechanism of NTP action, as it is a burgeoning therapeutic option for the treatment of cancer.

Abbreviations: AL, autophagolysosome; AP, autophagosome; CTRL, control; DFO, desferrioxamine (desferal); EEA1, early endosome antigen 1; FAC, ferric ammonium citrate; FtL, ferritin light chain; FtH, ferritin heavy chain; IRP, iron-regulatory protein; IRE, iron-responsive element; LAMP1, lysosomalassociated membrane protein 1; LC3B, microtubule-associated protein 1 light chain 3 beta; NTP, non-thermal plasma; PAS, preautophagic structure; ROS, reactive oxygen species; TfR1, transferrin receptor 1