Expression patterns of autophagy and lysosomal proteins in human normal brain and glioblastoma cell lines

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Introduction

Autophagy is a major intracellular pathway dedicated to a continuous renovation of long- lived or damaged proteins and cytoplasmic organelles [1]. LC3A, LC3B and p62 are the main proteins that are extensively used for the study of autophagy [2]. Also, LAMP2A and Cathepsin D lysosomal proteins are important determinants of the auto-lysosomal flux. In this study, we investigate the expression patterns of these proteins in normal brain and glioblastoma cell lines.

Materials and methods

T98G and U87MG glioblastoma cell lines were used for these experiments. Cell lines were cultured under standard conditions in a 5% CO2 incubator at 37^{o} C and lysed with Ripa buffer (Sigma-Aldrich, Cat.No. R0278) with the complete mini protease inhibitor cocktail (Roche Diagnostics, GmbH) and phosphatase inhibitor cocktail (Cell Signaling Technology, Inc.).

Immunobloting

Proteins of each lysate (30μg) were resolved by discontinuous SDS gels using 10%, 12,5% and 7,5% separating and 5% staking gels and transferred to a PVDF membrane (pore size: 0.45μm, Millipore Corp., Cat.No. IPVH00010). All different experimental condition lysates were loaded on five different gels and transferred on five different membranes. Following blocking with TBS (pH 7.5) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dried milk for 2h at room temperature, membranes were hybridized at 4°C overnight with primary antibodies to: i) LC3A (1:30000,ab62720, Abcam), ii) LC3B (1:100,5F10, Nanotools), iii) LAMP2A (ab18528, Abcam), iv) Cathepsin D (ab6313, Abcam) and v)p62 (1:5000,ab64134, Abcam).The membranes were then hybridized for 2 h at room temperature with the secondary a)sheep polyclonal antibody to rabbit IgG (H + L)-HRP Conjugate (1:10,000; Dako, PO163) and b) goat polyclonal antibody to rabbit IgG (H + L)-HRP Conjugate at a dilution of 1:15,000(Biorad; The images of the blots were captured utilizing Chemidoc™ MP imaging system (Biorad), and they were analysed by the accompanied Image Lab software.

Results

Immunobloting

In order to investigate the expression patterns of autophagy and lysosomal proteins we compared cell lines T98G and U87MG with human normal brain.

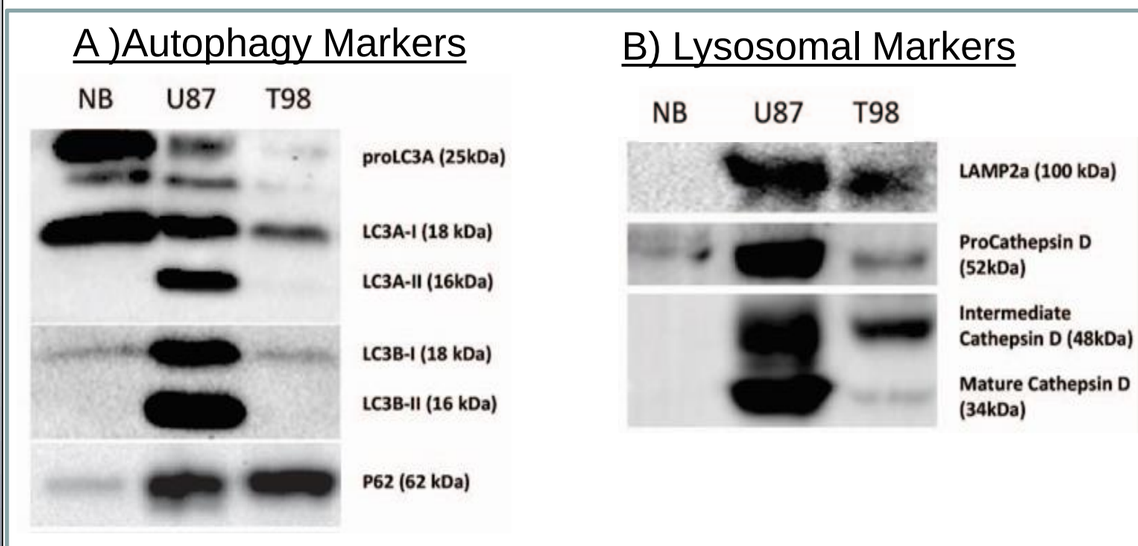

Figure 1: Western Blot analysis for (A) autophagy and (B) lysosomal markers in human normal brain and glioblastoma cell lines U87MG and T98G.

Western blot analysis showed a strong over-expression of pro LC3A and LC3A-I in normal brain, contrary to LC3A-II and LC3B that appears a weak expression. LC3A–II and LC3B were highly expressed in U87MG, but not inT98G. Both p62 and lysosomal markers showed intense expression in glioblastoma cell lines compared to normal brain.

Immunohisrochemistry for Autophagy Proteins LC3A-LC3B under Exposure to Different Stresses.

Cultured cell lines under oxygenated conditions and optimal culture medium were placed at poly-L-lysin coated slides and immunohistochemistry for LC3A and LC3B was performed. To examine whether and which of the tumor microenvironment stress factors are able to induce LC3 and the SLS phenomenon, cultures were performed under glucose metabolism blockage with 2-deoxy-D-glucose and low pH. Exposure to 2DG resulted in dramatic increase of diffuse LC3 expression and accumulation of large autophagic vacuoles similar to the SLS, in both cell lines. Low pH induced SLS in the U87 cell line but not in the T98 one.

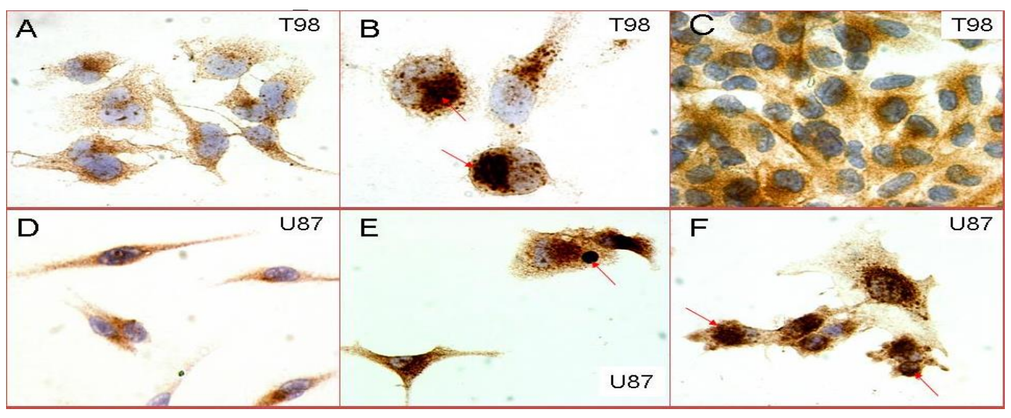

Figure 2: T98G in optimal culture condition (A), under exposure to 2DG (B) and low pH (C), and U87MG in optimal culture conditions (D), under exposure to 2DG (E) and low pH (F).

Confocal Microscopy

In order to assess whether LC3A and LC3B autophagosomes are distinct or overlap, double immunostaining with LC3A and LC3B antibodies was performed with confocal microscopy assessment in T98G and U87MG cell lines, both in optimal culture condition and under exposure to bafilomycin A. Images revealed that autophagosomes were stained either with LC3A or LC3B with apparent lack of co-localization,

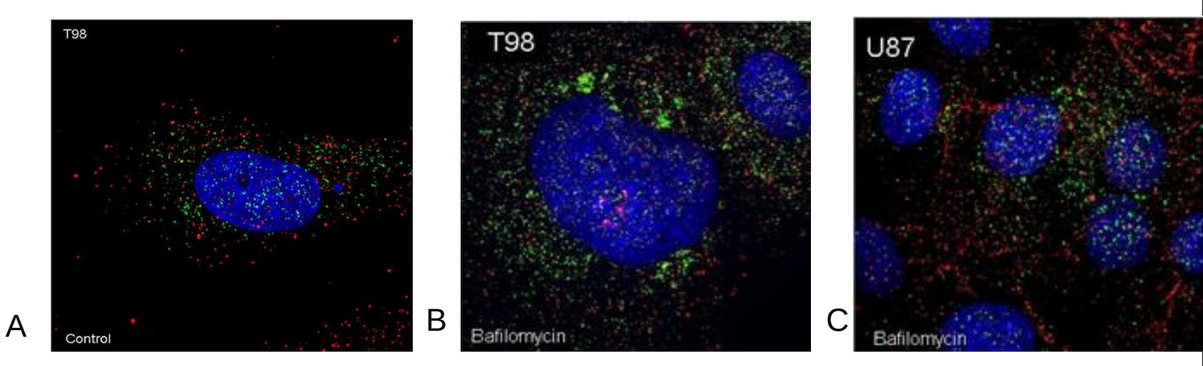

Figure 3: Confocal images of LC3A- LC3B in T98G cell line :(A) in optimal culture conditions and (B) under exposure to bafilomycin A and U87MG (C) under exposure to bafilomycin A.

Conclusion

- LC3A-I, unlike LC3B, has a strong expression in human normal brain. Lysosomal markers and p62 is poorly expressed in normal brain compared to glioblastoma cells. Also, both LC3A and B, as well as the lysosomal markers are overexpressed in the U87MG cells compared to T98.
- Of interest, the lack of LC3A and LC3B co-localization in confocal microscopy, implies a different biogenesis of LC3A and LC3B autophagosomes.
- Finally, LC3 and SLS were evident in glioblastoma cell lines and were inducible under exposure to 2DG and low pH.

References

1.Klionsky DJ and Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science,* 2000, 290: 1717-1721.

2. Kabeya Y, Mizushima N, Ueno T *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 2000;19, 5720-5728.