

Mesenchymal Stem Cells

AUTOPHAGY: CELL DEATH OR SURVIVAL? INTERACTION WITH OTHER STRESS PATHWAYS

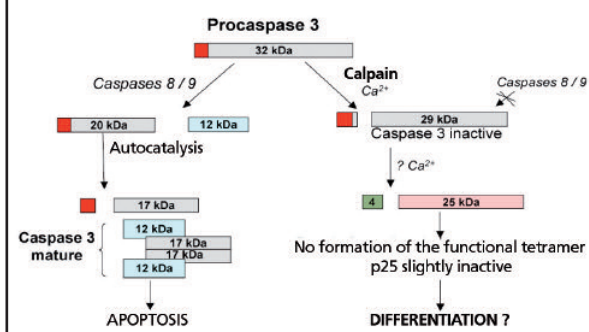
Oliver L., Hue E., Vallette F. M. Center for Cancer Research Nantes – Angers INSERM UMR 892, University of Nantes, 9 Quai Moncoussu, 44035 Nantes Cedex 01, France

INTRODUCTION

AUTOPHAGY is a regulated process of the degradation and recycling of cellular constituents, participating in organelle turnover and in the bioenergetics of starvation. Taken to the extreme, autophagy could ultimately result in cell death, through excessive self-digestion and degradation of essential cellular constituents. Thus, it is unclear whether autophagy is fundamentally a cell survival or a cell death pathway – or both.

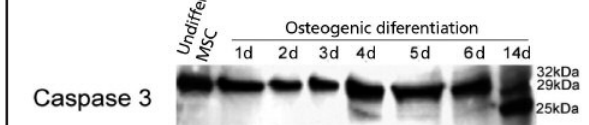
The aim of this work is to investigate the role of Bcl-XL in autophagy and apoptosis in Mesenchymal Stem Cells. Mesenchymal Stem Cells or MSC are generally resistant to apoptosis but exhibit high levels of autophagy. Once differentiated these cells become sensitive to apoptosis and little or no autophagy is observed.

Cleavage of Caspase-3 in MSCs: Apoptosis versus Differentiation

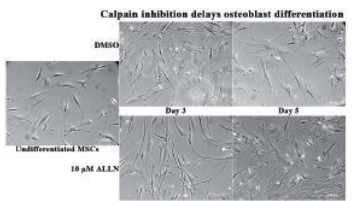


During osteogenic differentiation of MSCs, Caspase-3 is cleaved first into 29 kDa (Pelletier et al, 2005) and then into 25 kDa.

Pelletier, M., Oliver, L., Meflah, K. and Vallette, F.M. (2005). Caspase-3 can be pseudo-activated by a Ca^{2+} -dependent proteolysis at a non-canonical site. *FEBS Lett.* 579, 2364-2368.

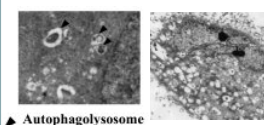


Using ALLN (a Calpain inhibitor I) this cleavage of Caspase-3 is prevented and differentiation is delayed. This suggests that Calpain could be implicated in the activation of Caspase-3 during differentiation.

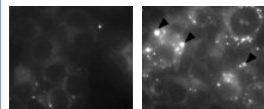


Autophagy activity detection

Transmission electron microscopy



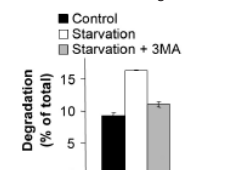
MDH labelling of autophagosomes



Control Starvation

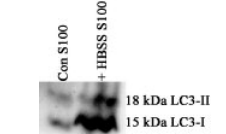
Measurement of long-lived protein degradation using pulse chase ^{14}C .

We induce autophagy with HBSS. The % of degraded proteins is compared to the total amount of proteins in cells (addition of 3MA methylamine, an inhibitor for degradation).



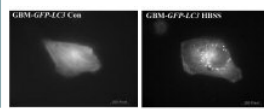
Detection by Western blot: cleavage of LC3-B (ATG-8) into LC3-BI and LC3-BII.

Western blot of MSC cultured in the absence or in the presence of HBSS. Then autophagy was quantified by the appearance of the cleavage of ATG-8 (LC3B). MSC cells are autophagy competent.

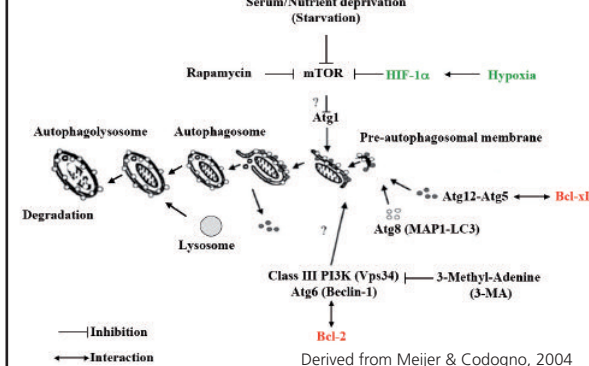


Sigma anti-LC3-B ref: L7543

Labelling of autophagosomes after transfection with GFP-LC3-B



Autophagy Pathway



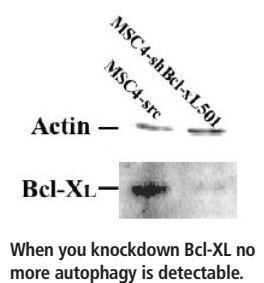
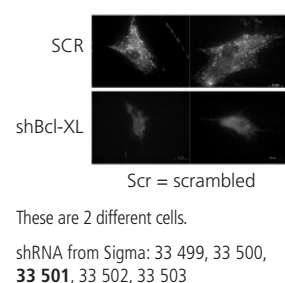
The role of Bcl-XL in autophagy in MSCs.

The essential autophagy protein, Beclin-1 (ATG 6) binds to and is inhibited by Bcl-2 or Bcl-XL. This interaction involves the Bcl-2 homology 3 (BH3) domain in Beclin-1 and the BH3 binding groove of Bcl-2/Bcl-XL.

Nutrient starvation, a potent physiologic inducer of autophagy, can stimulate the dissociation of Beclin-1 and Bcl-xL/Bcl-2. Thus the Bcl-2 family of proteins initially characterised as regulators of apoptosis should, in light of these data, also regulate autophagy.

Effect of Bcl-XL knock-down on autophagic response

Effect of Bcl-XL knock-down (note that little or no Bcl-2 is detectable in MSCs) MSCs were infected with shBcl-XL or src viral particles. 48 h later total cell extracts were made and Western Blot determined the expression of Bcl-XL. The efficiency of the knockdown of Bcl-XL was about 80% (average of 3 experiments) as compared to an internal control actin.



CONCLUSION

In MSCs, surprisingly Bcl-XL stimulated the autophagic activity in cells, which could be qualified by an increase in the number and size of the autophagosomes. The knock-down of Bcl-XL completely inhibited autophagic activity and sensitize these cells to apoptotic insults. Finally, in MSCs, autophagy appears to be the predominant stress sensor mechanism, and is likely to be used as a protection against apoptotic insults.

Induction of Apoptosis by Time Lapse Video-microscopy analysis

Time Lapse of MSC cells cultured in HBSS. Addition of growth factors

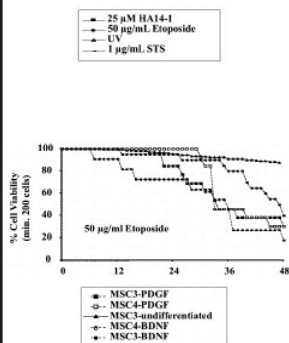
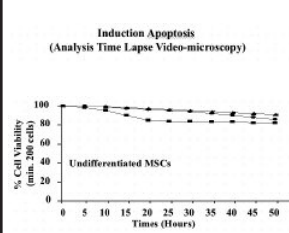
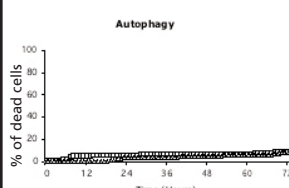


Fig above: We induced apoptosis by 4 different insults: HA14-1 (induces apoptosis in cells), Etoposide (targets the nucleus, a chemotherapeutic agent), UV irradiation (cells die by apoptosis after 24h), Sterosporin (broad inhibitor of protein kinases, cell death after 16h by apoptosis) We see here that the cells don't die.

Fig below: MSCs induced differentiation along the neuro-pathway PDGF induces differentiation to glial cells, BDNF differentiates along the neuronal pathway.

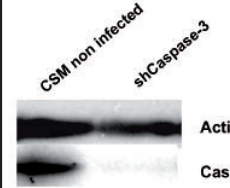
Once you induce differentiation with Etoposide, the cells become sensitive to apoptosis.

Reference HA14-1 Oliver, L., Mahe, B., Gree, R., Vallette, F.M. and Jun, P. (2007). HA14-1, a small molecule inhibitor of Bcl-2, bypasses chemoresistance in leukaemia cells. *Leuk. Res.* 31, 859-863.



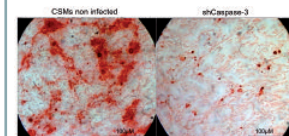
MSCs were cultured in the presence of HBSS and the cells were photographed every 10 min over 72 h. The number of dead cells was quantified at each point. The graphs depict the results from 3 different experiments: 3 different cells of MSCs. We see here that autophagy is not accompanied by cell death.

Caspase-3 knock-down by shRNA



MSCs are cultured over 3 weeks in the presence of beta-glycerophosphate, dexamethasone and ascorbic acid to induce osteogenesis. shRNA from Sigma 10798UTR: 3749, 3550, 3551, 3552 (lentiviral particles)

Alizarin Red S staining

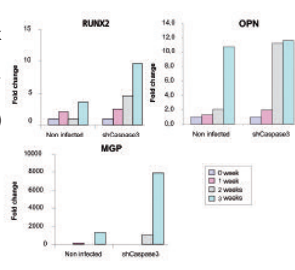


Alizarin Red S stains mineral nodules composed of calcium produced by osteoblasts. These are present during terminal osteogenic differentiation. Knock-down of Caspase-3 delays terminal osteogenic differentiation.

RT-qPCR analysis of osteogenic markers during differentiation.

MSCs are not differentiated, there are no markers for osteoblasts. If we block Caspase-3 this differentiation is disrupted. If we add growth factors and block Caspase-3, the cells try other ways to induce differentiation: the genes for osteogenic differentiation RUNX2, OPN and MGP are activated. (indirect results)

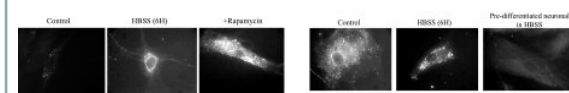
We are working to see what the direct targets of Caspase-3 are.



There seems to be another mechanism, which compensates the absence of Calpain during differentiation.

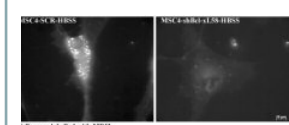
MDH staining of MSCs

MSC-Bcl-XL cells and MSC-scr cells were cultured in HBSS for 6 h to induce autophagy in these cells. The cells were cultured for the last 30 min in the presence of 1 μM Monodansyl pentane (MDH) to label autophagosomes. At the end of the incubation, the cells were washed with PBS then analysed under a microscope at Ex: 335 nm/Em: 525 nm.



High basal autophagy in MSCs, which can be amplified by starvation or inhibition of mTOR.

Loss of autophagic response after differentiation of MSCs.

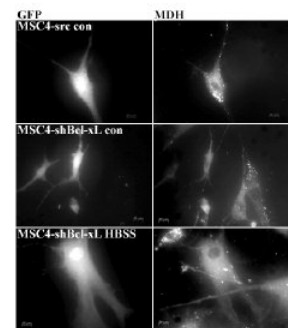
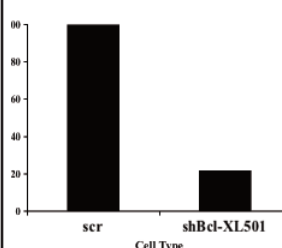


By adding HBSS to induce starvation, there is an increase of autophagy. In the absence of Bcl-XL you can not induce autophagy. (There is no basal autophagy in absence of Bcl-XL. These cells are no more autophagy competent).

Effect of Bcl-XL knock-down on autophagic response Confirmation of the results.

Since the knock-down in MSC cells affects between 60-80% of the cells, we need to confirm the results. Therefore knock-down was done using a pSilencer shRNA GFP-Bcl-XL. After transfection the cells were cultured in the presence or in the absence of HBSS for 6 h and the stained with MDH.

Graph of percentage knock-down of Bcl-XL.



MSC were infected with pSilencer GFP-Bcl-XL or a pSilencer GFP-scr. After 72 h the cells were incubated in HBSS and autophagy analysed by MDH staining. At the same time the cells were reinfected with Bcl-XL using a PTD-Bcl-XL construct. (The PTD sequence YGRKKQRR is an internalisation sequence, which allows the protein to be taken up by the cells). The results obtained show that in the absence of Bcl-XL there are little or no autophagosomes and after the treatment with PTD-Bcl-XL the cells recuperated their autophagosomal activity.