Mechanisms of Ageing and Development 177 (2019) 88–90

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

Short communication

In situ evidence of cellular senescence in Thymic Epithelial Cells (TECs) during human thymic involution

Alexandra Barbouti[a,1], Konstantinos Evangelou[a,b,*,1], Ioannis S. Pateras[b], Alexandra Papoudou-Bai[c], Amalia Patereli[d], Kalliopi Stefanaki[d], Dimitra Rontogianni[e], Daniel Muñoz-Espín[f], Panagiotis Kanavaros[a], Vassilis G. Gorgoulis[b,g,h,*]

[a] Department of Anatomy-Histology-Embryology, Medical School, University of Ioannina, Ioannina, Greece
[b] Molecular Carcinogenesis Group, Department of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Athens, Greece
[c] Department of Pathology, Medical School, University of Ioannina, Ioannina, Greece
[d] Department of Pathology, Agia Sophia Hospital, Athens, Greece
[e] Department of Anatomic Pathology, Evangelismos General Hospital, University of Athens, Athens, Greece
[f] CRUK Cambridge Centre Early Detection Programme, Department of Oncology, University of Cambridge, Hutchison/MRC Research Centre, Cambridge, UK
[g] Faculty Institute for Cancer Sciences, Manchester Academic Health Sciences Centre, University of Manchester, Manchester, UK
[h] Biomedical Research Foundation, Academy of Athens, Athens, Greece

ARTICLE INFO

Keywords:
Thymic involution
Cellular senescence
SenTraGor™
Lipofuscin

ABSTRACT

Cellular senescence, an age-related process in response to damage and stress, also occurs during normal development and adult life. The thymus is a central lymphoepithelial organ of the immune system that exhibits age-related changes termed thymic involution. Since the mechanisms regulating thymic involution are still not well elucidated, we questioned whether cellular senescence is implicated in this process. We demonstrate, for the first time *in situ*, that cellular senescence occurs during human thymic involution using SenTraGor™, a novel chemical compound that is applicable in archival tissue material, providing thus further insights in thymus histophysiology.

Cellular senescence is a stress response mechanism that ensures cellular homeostasis. It is triggered by various signals during normal development, adult life and in the frame of various non age-related and age-related disorders (Bartkova et al., 2006; Burton and Krizhanovsky, 2014; Gorgoulis and Halazonetis, 2010; Halazonetis et al., 2008; Liontos et al., 2007; Liontos et al., 2009; Munoz-Espin and Serrano, 2014; Perez-Mancera et al., 2014; Rodier and Campisi, 2011; Salama et al., 2014). Senescent cells are metabolically active cells that simultaneously exhibit cell cycle arrest, increased beta-galactosidase (SA-β-gal) activity, a secretory function termed Senescence-Associated Secretory Phenotype (SASP) and accumulation of lipofuscin in the cytoplasm (Dimri et al., 1995; Evangelou and Gorgoulis, 2017; Evangelou et al., 2017; Georgakopoulou et al., 2013). DNA damage and heterochromatin foci (SAHFs) are frequently observed in the nucleus of senescent cells (Burton and Krizhanovsky, 2014; Munoz-Espin and Serrano, 2014; Rodier and Campisi, 2011; Salama et al., 2014; Sharpless and Sherr, 2015).

The human thymus is a cardinal organ of the immune system implicated in the production of effector and regulatory T cells. T lymphocytes that leave the thymus are functional as they can recognize antigens in the self major histocompatibility complex (MHC) (Bai et al., 2013; Bronietzki et al., 2015; Hernandez et al., 2010; Kanavaros et al., 2001; Klein et al., 2011; Papoudou-Bai et al., 2012; Rezzani et al., 2008). The organ undergoes an age-related regression in its size, initiated prior to early puberty, termed thymic involution (Shanley et al., 2009). Several causes and valid hypotheses involving immunological or metabolic rationales have been proposed to explain this phenomenon (Dooley and Liston, 2012). Thymic involution, results in the decline of the output of naïve T cells and the shrinkage of T cell repertoire at the periphery (Aspinall and Mitchell, 2008; Chinn et al., 2012; Montecino-

Abbreviations: AP, alkaline phosphatase SenTraGor™; GL13, Sudan Black-B analogue; DAB, diaminobenzidine; SASP, senescence-associated secretory phenotype; TECs, thymic epithelial cells; FFPE, formalin fixed paraffin embedded; SAHF, senescence-associated heterochromatin foci; DDR, DNA damage response; 8-Oxo, 8-oxoguanine; MHC, major histocompatibility complex

* Corresponding authors at; Vassilis G. Gorgoulis: Molecular Carcinogenesis Group, Department of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Athens, Greece; Konstantinos Evangelou: Department of Anatomy-Histology-Embryology, Medical School, University of Ioannina, Ioannina, Greece.
E-mail addresses: kostasevangelou@cc.uoi.gr (K. Evangelou), vgorg@med.uoa.gr (V.G. Gorgoulis).
[1] Equal contribution.

https://doi.org/10.1016/j.mad.2018.02.005
Received 18 December 2017; Received in revised form 21 February 2018; Accepted 23 February 2018
Available online 25 February 2018
0047-6374/

Rodriquez et al., 2005; Ponnappan and Ponnappan, 2011; Rezzani et al., 2008; Taub and Longo, 2005).

Given that cellular senescence is a hallmark of aging (Bartkova et al., 2006; Gorgoulis and Halazonetis, 2010; Halazonetis et al., 2008; Herbig et al., 2006; Lopez-Otin et al., 2013), we questioned whether cellular senescence is implicated in age-related human thymic involution. We, therefore, collected formalin fixed and paraffin embedded (FFPE) thymic tissues from a total of 27 individuals: 3 infants (age range: 51 days–6.5 months), 8 children (age range: 13 months–10 years), 6 adolescent cases (age range: 11–14 years) and 10 adults (age range: 34–73 years). The retrospective experimental use of FFPE material was approved by the Bio-Ethics Committee of Medical School of Athens, in accordance with the Declaration of Helsinki and local laws and regulations. This archival material could not be investigated for detection of senescent cells since the most widely used method, detection of the SA-β-gal activity, is not applicable in FFPE material (Dimri et al., 1995). In order to deal with this issue, we designed, generated and validated an innovative biotinylated Sudan Black-B (SBB) based chemical compound (GL13, Evangelou et al., 2017), that is commercially available under the trademark SenTraGor™ (Arriani Pharmaceuticals S.A., Greece, Cat Number: AR8850040 and AR8850080). The novel chemical analogue is highly lipophilic and binds to lipofuscin (mimicking SBB), a cytoplasmic hallmark of senescent cells. This interaction can be visualized via a hybrid histochemical/immunohistochemical method, allowing thus recognition of senescent cells in any biological material, irrespectively of its processing (Evangelou et al., 2017). In the current study, this methodology was performed in FFPE thymic tissues. Double staining resulting in two discrete chromogenic products is also feasible with this methodology, as previously shown (Evangelou et al., 2017).

Interestingly, we detected a number of Thymic Epithelial Cells (TECs) mainly in the vicinity of Hassall's corpuscles that exhibited dense cytoplasmic SenTraGor™ positivity in all adolescent cases ((labeling index: 4–8%) and in all aged thymuses (labeling index: 10–17%) while SenTraGor™ staining was not detected in infants and children (Fig. 1a–d). These findings imply that cellular senescence occurs in more advanced stages of human thymic involution. Positive SenTraGor™ TECs and stromal cells showed simultaneously nuclear $p21^{WAF1/Cip1}$ immunopositivity in double staining experiments (Fig. 1e), a finding further denoting their senescent nature. Senescent TECs in the adult cases stained at the same time positively for the DNA Damage response (DDR) marker γ-H2AX (Fig. 1f). 8-oxoguanine (8-oxo-G) immunopositivity, a marker of oxidative stress was evident in the adults (Fig. 1g), while both markers were negative in the adolescent cases (Fig. 1h). The latter finding indicates that oxidative stress driven DNA damage activates senescence during human thymic involution exclusively in the elderly. Similarly, increased β-galactosidase activity and γ-H2AX immunopositivity were reported in mouse TECs with increasing age (Aw et al., 2008). A sizeable proportion of TECs expressed the cyclin dependendent kinase inhibitors $p16^{INK4A}$ and $p21^{WAF1/Cip1}$, while these cells exhibited very low/null proliferation as evidenced by Ki67 staining and absence of apoptotic markers such as cleaved caspase-3 (Bai et al., 2013; Kanavaros et al., 2001). Increase in senescent lymphocytes, previously shown in mice (senescence associated CD4+ T cells) during thymic involution, was not observed in the examined clinical settings, implying species specific variations (Sato et al., 2017).

Our *in situ* findings support a putative role of cellular senescence at later phases of human thymic involution, a phenomenon never shown so far in clinical settings, to the best of our knowledge. Our results also imply different, age dependent causes that putatively trigger senescence in a cumulative manner during this process. During adolescence, cellular senescence seems to be activated by various factors in the frame of an evolutionary program, as also proposed by the immunological scenarios (Dooley and Liston, 2012). According to the latter, thymic involution seems to be a response against threats related to continuous thymocyte differentiation that can eventually cause improper "shaping" and function of the immune cell repertoire. It is tempting to speculate that senescent TECs, through SASP, may be implicated in the selection of the T cell population during young-adult life, an evolutionary process optimized for fighting infections and avoiding reaction to self antigens (Dowling and Hodgkin, 2009; Dooley and Liston, 2012). In more advanced ages, and in line with the metabolic rationale, senescence in TECs seems to be part of an oxidative DNA damage-driven degenerative process (Shanley et al., 2009; Dooley and Liston, 2012). In this context,

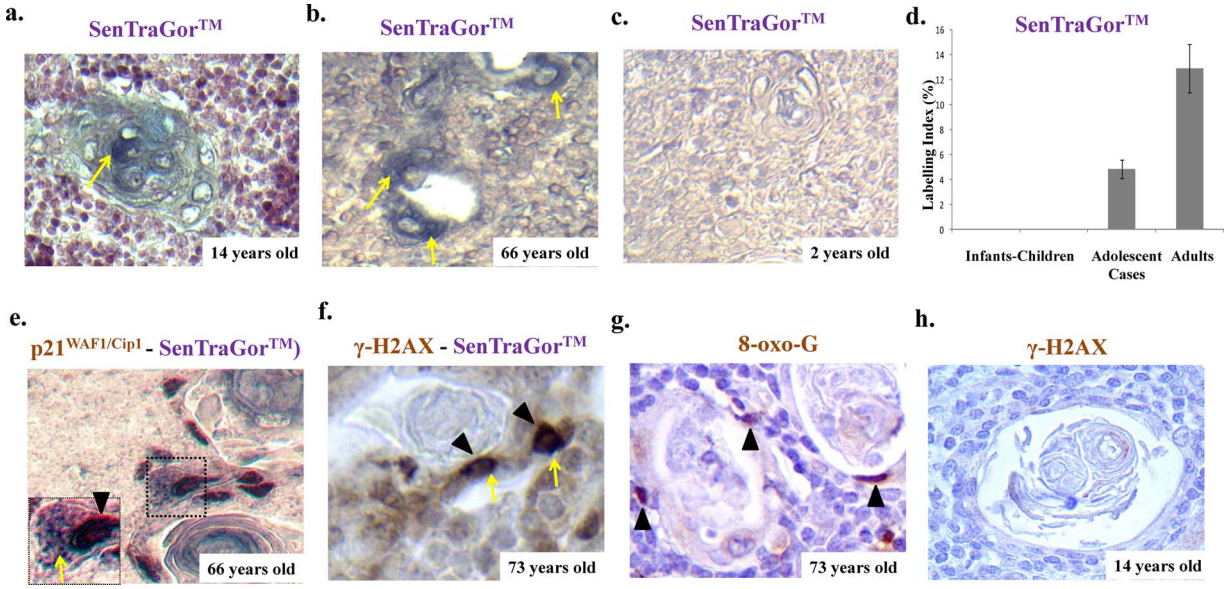

Fig. 1. Representative images from the hybrid Histo (SenTraGor™)-Immunohistochemical staining in human thymic tissues, depicting positive senescent TECs in a representative adolescent (a) and adult case (b). No SenTraGor™ reaction was observed in infants and children as shown in the representative case (c). Graph depicts SenTraGor™ staining results (mean % labeling index) in the 27 examined thymic cases (infants/children: n = 11, adolescent cases: n = 6 and adults: n = 10; bars = standard deviations, SD). (d). Double staining experiments showed concurrent positivity of senescent cells with SenTraGor™ in the cytoplasm and for $p21^{WAF1/Cip1}$ within the nucleus (e). Double SenTraGor™/γ-H2AX positivity (f) and 8-oxoguanine (8-oxo-G) immunoreactivity (g) in TECs of a representative adult case. γ-H2AX immunostaining is absent in TECs of the adolescent cases (h). [Alkaline Phosphatase (AP) chromogenic reaction results in a dark blue cytoplasmic product: yellow arrows]. [Diaminobenzidine (DAB) reaction results in a nuclear brown color reaction: black arrowheads]. Magnifications; a–c: x200, e,f: x:630, g–h: x400.

TAp63 up-regulation might be implicated in inducing senescence in TECs (Burnley et al., 2013). Additionally, immunosenescence that occurs with age and is accompanied by altered gene expression and epigenetic regulation influences clearance and senescent TEC accumulation (Sidler et al., 2013). Although TECs are of epithelial origin, they exhibit some features reminiscent of the stromal cells in other organs. Induction of senescence in TECs during thymic involution is in line with our previous concept that "stromal" cells prefer to enter stress-induced senescence (Georgakopoulou et al., 2016). Finally, the exact role of cellular senescence in thymic histophysiology and pathology can now be further investigated in fixed material, using SenTraGor™.

Conflict of interest

The authors wish to declare no conflict of interest.

Acknowledgments

We would like to thank Associate Professor Dina Tiniakos for providing material for this investigation. This work was financially supported by the "SYNTRAIN" ITN Horizon 2020 Grant No DHI GLOBAL EUROPE and NKUASARG grants 70/3/12128, 70/3/8916, 70/3/1135.

References

Aspinall, R., Mitchell, W., 2008. Reversal of age-associated thymic atrophy: treatments, delivery, and side effects. Exp. Gerontol. 43, 700–705.
Aw, D., Silva, A.B., Maddick, M., von Zglinicki, T., Palmer, D.B., 2008. Architectural changes in the thymus of aging mice. Aging Cell 7, 158–167.
Bai, M., Doukas, M., Papoudou-Bai, A., Barbouti, A., Stefanaki, K., Galani, V., Kanavaros, P., 2013. Immunohistological analysis of cell cycle and apoptosis regulators in thymus. Ann. Anat. 195, 159–165.
Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C.L., Dyrskjot, L., Orntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T.D., Bartek, J., Gorgoulis, V.G., 2006. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 444, 633–637.
Bronietzki, A.W., Schuster, M., Schmitz, I., 2015. Autophagy in T-cell development, activation and differentiation. Immunol. Cell Biol. 93, 25–34.
Burnley, P., Rahman, M., Wang, H., Zhang, Z., Sun, X., Zhuge, Q., Su, D.M., 2013. Role of the p63-FoxN1 regulatory axis in thymic epithelial cell homeostasis during aging. Cell. Death. Dis. 21 (4), e932.
Burton, D.G., Krizhanovsky, V., 2014. Physiological and pathological consequences of cellular senescence. Cell. Mol. Life Sci. 71, 4373–4386.
Chinn, I.K., Blackburn, C.C., Manley, N.R., Sempowski, G.D., 2012. Changes in primary lymphoid organs with aging. Semin. Immunol. 24, 309–320.
Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., Campisi, J., 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. U. S. A. 92, 9363–9367.
Dooley, J., Liston, A., 2012. Molecular control over thymic involution: from cytokines and microRNA to aging and adipose tissue. Eur. J. Immunol. 42, 1073–1079.
Dowling, M.R., Hodgkin, P.D., 2009. Why does the thymus involute? A selection-based hypothesis. Trends Immunol. 30, 295–300.
Evangelou, K., Gorgoulis, V.G., 2017. Sudan black B, the specific histochemical stain for lipofuscin: a novel method to detect senescent cells. Methods Mol. Biol. 1534, 111–119.
Evangelou, K., Lougiakis, N., Rizou, S.V., Kotsinas, A., Kletsas, D., Munoz-Espin, D., Kastrinakis, N.G., Pouli, N., Marakos, P., Townsend, P., Serrano, M., Bartek, J., Gorgoulis, V.G., 2017. Robust, universal biomarker assay to detect senescent cells in biological specimens. Aging Cell 16, 192–197.
Georgakopoulou, E.A., Tsimaratou, K., Evangelou, K., Fernandez Marcos, P.J., Zoumpourlis, V., Trougakos, I.P., Kletsas, D., Bartek, J., Serrano, M., Gorgoulis, V.G., 2013. Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence: a method applicable in cryo-preserved and archival tissues. Aging (Albany NY) 5, 37–50.
Georgakopoulou, E., Evangelou, K., Havaki, S., Townsend, P., Kanavaros, P., Gorgoulis, V.G., 2016. Apoptosis or senescence? Which exit route do epithelial cells and fibroblasts preferentially follow? Mech. Ageing Dev. 156, 17–24.
Gorgoulis, V.G., Halazonetis, T.D., 2010. Oncogene-induced senescence: the bright and dark side of the response. Curr. Opin. Cell Biol. 22, 816–827.
Halazonetis, T.D., Gorgoulis, V.G., Bartek, J., 2008. An oncogene-induced DNA damage model for cancer development. Science 319, 1352–1355.
Herbig, U., Ferreira, M., Condel, L., Carey, D., Sedivy, J.M., 2006. Cellular senescence in aging primates. Science 311, 1257.
Hernandez, J.B., Newton, R.H., Walsh, C.M., 2010. Life and death in the thymus–cell death signaling during T cell development. Curr. Opin. Cell Biol. 22, 865–871.
Kanavaros, P., Stefanaki, K., Rontogianni, D., Papalazarou, D., Sgantzos, M., Arvanitis, D., Vamvouka, C., Gorgoulis, V., Siatitsas, I., Agnantis, N.J., Bai, M., 2001. Immunohistochemical expression of p53, p21/waf1, rb, p16, cyclin D1, p27, Ki67, cyclin A, cyclin B1, bcl2, bax and bak proteins and apoptotic index in normal thymus. Histol. Histopathol. 16, 1005–1012.
Klein, L., Hinterberger, M., von Rohrscheidt, J., Aichinger, M., 2011. Autonomous versus dendritic cell- dependent contributions of medullary thymic epithelial cells to central tolerance. Trends Immunol. 32, 188–193.
Liontos, M., Koutsami, M., Sideridou, M., Evangelou, K., Kletsas, D., Levy, B., Kotsinas, A., Nahum, O., Zoumpourlis, V., Kouloukoussa, M., Lygerou, Z., Taraviras, S., Kittas, C., Bartkova, J., Papavassiliou, A.G., Bartek, J., Halazonetis, T.D., Gorgoulis, V.G., 2007. Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. Cancer Res. 67, 10899–10909.
Liontos, M., Niforou, K., Velimezi, G., Vougas, K., Evangelou, K., Apostolopoulou, K., Vrtel, R., Damalas, A., Kontovazenitis, P., Kotsinas, A., Zoumpourlis, V., Tsangaris, G.T., Kittas, C., Ginsberg, D., Halazonetis, T.D., Bartek, J., Gorgoulis, V.G., 2009. Modulation of the E2F1-driven cancer cell fate by the DNA damage response machinery and potential novel E2F1 targets in osteosarcomas. Am. J. Pathol. 175, 376–391.
Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., Kroemer, G., 2013. The hallmarks of aging. Cell 153, 1194–1217.
Montecino-Rodriquez, E., Min, H., Dorshkind, K., 2005. Reevaluating current models of thymic involution. Semin. Immunol. 17, 356–361.
Munoz-Espin, D., Serrano, M., 2014. Cellular senescence: from physiology to pathology. Nat. Rev. Mol. Cell Biol. 15, 482–496.
Papoudou-Bai, A., Bai, M., Doukas, M., Stefanaki, K., Spyridonos, P., Karatzias, G., Barbouti, A., Galani, V., Kanavaros, P., 2012. Immunohistological characterization of thymic dendritic cells. In Vivo 26, 985–992.
Perez-Mancera, P.A., Young, A.R., Narita, M., 2014. Inside and out: the activities of senescence in cancer. Nat. Rev. Cancer 14, 547–558.
Ponnappan, S., Ponnappan, U., 2011. Aging and immune function: molecular mechanisms to interventions. Antioxid. Redox Signal. 14, 1551–1585.
Rezzani, R., Bonomini, F., Rodella, L.F., 2008. Histochemical and molecular overview of the thymus as site for T-cells development. Prog. Histochem. Cytochem. 43, 73–120.
Rodier, F., Campisi, J., 2011. Four faces of cellular senescence. J. Cell Biol. 192, 547–556.
Salama, R., Sadaie, M., Hoare, M., Narita, M., 2014. Cellular senescence and its effector programs. Genes Dev. 28, 99–114.
Sato, K., Kato, A., Sekai, M., Hamazaki, Y., Minato, N., 2017. Physiologic thymic involution underlies age-Dependent accumulation of senescence-associated CD4 + T cells. J. Immunol. 199, 138–148.
Shanley, D.P., Aw, D., Manley, N.R., Palmer, D.B., 2009. An evolutionary perspective on the mechanisms of immunosenescence. Trends Immunol. 30, 374–381.
Sharpless, N.E., Sherr, C.J., 2015. Forging a signature of in vivo senescence. Nat. Rev. Cancer 15, 397–408.
Sidler, C., Wóycicki, R., Ilnytskyy, Y., Metz, G., Kovalchuk, I., Kovalchuk, O., 2013. Immunosenescence is associated with altered gene expression and epigenetic regulation in primary and secondary immune organs. Front. Genet. 18 (4), 211.
Taub, D.D., Longo, D.L., 2005. Insights into thymic aging and regeneration. Immunol. Rev. 205, 72–93.