Autophagy induces protein carbamylation in fibroblast-like synoviocytes from patients with rheumatoid arthritis

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Abstract

Objectives. Autophagy is a homeostatic and physiological process that promotes the turnover of proteins and organelles damaged in conditions of cellular stress. We previously demonstrated that autophagy represents a key processing event creating a substrate for autoreactivity, which is involved in post-translational changes and generation of citrullinated peptides, recognized by the immune system in RA. In this study, we analysed whether autophagy is involved in other post-translational changes that can generate autoantigens, focusing on carbamylation processes. Carbamylation is a nonenzymatic post-translational modification, in which homocitrulline is generated by the reaction of cyanate with the primary amine of lysine residues; carbamylated peptides may accumulate during inflammation conditions.

Methods. The role of autophagy in the generation of carbamylated proteins was evaluated in vitro in fibroblasts as well as in synoviocytes from RA patients, treated with 5 µM tunicamycin or 200 nM rapamycin; the correlation between autophagy and carbamylated proteins was analysed in mononuclear cells from 30 naïve early-active RA patients.

Results. Our results demonstrated that cells treated with tunicamycin or rapamycin showed a significant increase of carbamylated proteins. Immunoblotting and immunoprecipitation experiments identified vimentin as the main carbamylated protein. Furthermore, a correlation was found between autophagy and carbamylation levels in mononuclear cells of naïve RA patients.

Conclusion. These data indicate that autophagy is able to induce in vitro carbamylation processes, and in vivo appears to be related to an increase in carbamylation during RA. These observations introduce a new pathogenetic mechanism of disease, which could contribute to more accurate monitoring of patients.

Key words: carbamylation, autophagy, rheumatoid arthritis, vimentin

Rheumatology key messages

- Autophagy induces protein carbamylation in fibroblast-like synoviocytes.
- Immunoprecipitation experiments identified vimentin as the main carbamylated protein.
- A significant association between autophagy and carbamylation levels was observed in mononuclear cells of naïve RA patients.

Introduction

RA is a systemic, inflammatory chronic disease characterized by a persistent immune response with inflammation and progressive joint destruction [1]; genetic and environmental factors have been implicated as having a role in disease development [2].

The aetio-pathogenic mechanisms include an interaction between the innate and adaptive immune response that involves antigen-presenting cells, the formation of autoreactive T cells and the production of autoantibodies,
such as RF and ACPAs [3]. Thus, RA may be considered a prototype of autoimmune disease, with the hallmarks of synovial inflammation and presence of autoantibodies [4]. One of the pathological features of RA is angiogenesis in the synovial tissues, which causes synovitis, cartilage destruction and pannus growth [5]. The synovial tissues in RA consist of excessive proliferated synovial cells and many kinds of infiltrated inflammatory cells [6].

Autophagy, which is a homeostatic and physiological process that promotes the turnover of damaged proteins and organelles during cellular stress [7], may be responsible for post-translational changes of proteins, which may alter their antigenicity [8], thus playing a role in the pathogenesis of RA [9]. During autophagy, parts of cytoplasm and organelles are encapsulated in double-membraned vacuoles, termed autophagosomes, which fuse with lysosomes to be degraded [7]. In a previous study we demonstrated in vitro a role for autophagy in the citrullination process, revealing this mechanism as a possible trigger for ACPAs [9]. Protein citrullination is an enzymatic post-translational modification mediated by the peptidyl arginine deiminase enzymes in the presence of Ca2+ that converts amino acid residues of arginine into citrulline through a process of deimination [10]. Citrullination is involved in several physiological processes, including regulation of the immune system [11], and plays an important role in the plasticity of nervous system cells [12] and in inflammatory processes, including RA [13, 14].

In contrast, carbamylation is a nonenzymatic post-translational modification, in which homocitrulline residues are generated by the reaction of cyanate with the primary amine of lysine residues within polypeptide chains of proteins. The physiological role of carbamylation is unclear, but recent studies demonstrated for many disease states, an involvement of carbamylated protein accumulation in their pathology, that is, these modified proteins are reported to accumulate in conditions of uremia, inflammation and cigarette smoking [15–17].

Isocyanic acid is formed primarily by spontaneous cleavage of urea into ammonia and cyanate; the latter is then rapidly converted into its reactive form, isocyanic acid. Cyanate may also originate from the thiocyanate metabolism, since myeloperoxidase catalyses the oxidation of thiocyanate in the presence of hydrogen peroxide, which is responsible for alterations of structural and functional protein properties promoting post-translational modification, including carbamylation processes [18].

Despite the low concentration of cyanate, trace amount of carbamylation can be detected in healthy individuals [19]; elevated carbamylation was extensivly reported among patients with renal dysfunction and elevated blood urea nitrogen levels [20].

Research on post-translational modification of proteins has proved to be of great interest in recent years, because in autoimmune diseases, such as RA [9, 10, 13, 14, 21], SLE [22] and APS [23], they may generate different autoantigens [24].

In this regard, we suggest autophagy as a key mechanism involved in post-translational modifications [9] and autoantigens generation [25]. Many auto-antigenic epitopes are indeed of intracellular derivation [26] and autophagy can be relevant for loss of tolerance to intracellular molecules and in this way be responsible for the autoimmune process [27].

In this research, we evaluated in vitro the role of autophagy in the generation of carbamylated proteins, and ex vivo the correlation between autophagy and carbamylated proteins in naive early-active patients with RA.

Methods

Cell cultures and treatments

Primary human fibroblast cells, arising from skin biopsy, were prepared as previously described [28, 29]. The cells were obtained from biopsies of sun-protected forearm skin according to standard culture methods. All the donors gave their informed consent. All the analyses were performed on cells between the third and seventh passage of culture at nearly 90% confluence. Cells were grown in DMEM (Sigma-Aldrich, Milan, Italy) containing 10% fetal calf serum (Sigma-Aldrich, Milan, Italy).

Synovial tissue was drawn during total knee replacement of RA and OA patients after collecting their informed consent. The patients’ mean age was 73.25 years (range 73–79 years); 50% of patients were female. Fibroblast-like synoviocytes were isolated as described in a previous study [30] and cultured in DMEM: nutrient mixture F-12 (DMEM/F12, Sigma-Aldrich, Milan, Italy), containing 10% fetal calf serum (Sigma-Aldrich, Milan, Italy).

All culture media contained 100 U/ml penicillin and 10 mg/ml streptomycin and all the cell cultures were maintained under standard conditions at 37°C in a humified atmosphere, containing 5% CO2.

For autophagy induction, cells were treated with 5 μM tunicamycin (from Streptomyces lysosuperficus; TNC, Calbiochem, La Jolla, CA, USA) or with 200 nM rapamycin (Sigma-Aldrich, Milan, Italy), for different incubation times (5 min, 20 min, 1 h or 4 h) at 37°C.

Western blot analysis of carbamylated proteins

Primary human fibroblasts, fibroblast-like synoviocytes (untreated or treated with TNC 5 μM or rapamycin 200 nM for different incubation times at 37°C) and monocyte cells were lysed in lysis buffer, containing 1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4 and 75 U of aprotinin. The cell suspensions underwent Dounce homogenization (10 strokes). The lysates were centrifuged for 5 min at 1300 g to remove nuclei and large cellular debris. The protein content was determined by Bradford assay (Bio-Rad, Segrate, MI, Italy) and equal amounts of whole-extract proteins were subjected to 10% SDS–PAGE. The proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Segrate, MI, Italy). Membranes were blocked with 5% defatted dried milk in Tris-buffered saline, containing 0.05% Tween-20. Rabbit anti-Carbamyl-Lysine polyclonal antibodies (Abs) (CliniSciences, Nanterre, France) and horseradish peroxidase-conjugated anti-rabbit IgG Abs (Sigma-Aldrich, Milan, Italy) were used. Immunoreactivity
was assessed by the chemiluminescence reaction, using the Enhanced Chemiluminescence western blotting detection system (Amersham, Buckinghmashire, UK).

The anti-Carbamyl-Lysine antibody was stripped from the PVDF and the membrane was then reprobed with a rabbit anti-vimentin mAb (Cell Signaling, Danvers, MA, USA).

Immunoprecipitation of vimentin
Primary human fibroblasts and fibroblast-like synoviocytes, untreated or treated with 5 μM TNC or 200 nM rapamycin for 20 min and 4 h at 37°C, were lysed in lysis buffer as described above. To preclear nonspecific binding, cell-free lysates were mixed with protein G-acrylic beads (Sigma-Aldrich, Milan, Italy) and stirred by a rotary shaker for 2 h at 4°C. The supernatants were centrifuged (500 g for 1 min) and then immunoprecipitated with goat polyclonal anti-vimentin Abs (R&D Systems, Minneapolis, MN, USA) or with irrelevant IgG as a negative control plus protein G-acrylic beads. The immunoprecipitates were analysed by western blot using a rabbit anti-Carbamyl-Lysine Ab (CliniSciences, Nanterre, France) and immunoreactivity assessed by chemiluminescence reaction, as reported above. To confirm that the positive band was vimentin, the PVDF membrane was stripped and then reprobed with a rabbit anti-vimentin mAb (Cell Signaling, Danvers, MA, USA). Densitometric scanning analysis was performed by Mac OS X (Apple Computer International, Cupertino, CA, USA), using National Institutes of Health ImageJ 1.62 software. The density of each band (absolute value) in the same gel was analysed.

Analysis of autophagy
Primary human fibroblasts, fibroblast-like synoviocytes (untreated or treated with TNC 5 μM or rapamycin
200 nM at 37°C) and monocytic cells were lysed in lysis buffer as described above. The cell suspensions underwent Dounce homogenization (10 strokes) and the lysates were centrifuged for 5 min at 1300 g in order to remove nuclei and large cellular debris. Protein concentration of sample lysates was evaluated by Bradford assay (Bio-Rad, Segrate, MI, Italy). Then, the lysates were subjected to 15% SDS-PAGE and the proteins electrophoretically transferred onto PVDF membranes (Bio-Rad, Segrate, MI, Italy). Membranes were blocked with 5% defatted dried milk in Tris-buffered saline, containing 0.05% Tween-20 and probed with rabbit polyclonal anti-LC3 Ab (MBL International Corporation, Ottawa, Canada, USA) or with anti-actin mAb (Sigma-Aldrich, Milan, Italy). To visualize bound Abs, PVDF membranes were probed with horseradish peroxidase-conjugated anti-rabbit IgG Abs (Sigma-Aldrich, Milan, Italy) or anti-mouse IgG Abs (Sigma-Aldrich, Milan, Italy) and then immunoreactivity was assessed by chemiluminescence reaction, as reported above. Densitometric scanning analysis was performed by Mac OS X (Apple Computer International, Cupertino, CA, USA), using National Institutes of Health ImageJ 1.62 software. The density of each band of LC3-II and actin was analysed and the densitometric LC3-II/actin ratios are shown.

Patients

Thirty consecutive early RA patients, all naïve to treatments [10 males and 20 females, mean (s.d.) age 49 (14) years, mean disease duration 26 (15) weeks], attending the Rheumatology Unit at ‘Sapienza’ University of Rome and 20 healthy donors [9 men and 11 women, mean (s.d.) age 45 (11) years] were enrolled. All the patients fulfilled the 2010 ACR/EULAR criteria [31]. Patients were recruited after written informed consent. Exclusion criteria were: previous use of steroids and/or DMARDs, cardiovascular disease, cancer, diabetes mellitus, metabolic syndrome, renal failure and IBD.

The mean (s.d.) tender joint count of patients with RA was 5.3 (4.4); the mean (s.d.) swollen joint count was 3.4 (3.6); the DAS 28 was 3.36 (1); the ESR was 37.7 (30) mmHg/h; and the CRP was 17.6 (26) mg/l. Eighteen patients were positive for RF, 21 for anti-CCP antibodies and 9 for anti-nuclear antibodies. This study was approved by the local ethical committee of the ‘Sapienza’ University of Rome.

Monocyte isolation

Peripheral blood mononuclear cells from the venous blood of naïve patients with early-active RA and healthy
donors were isolated by Lymphoprep density-gradient centrifugation (Nycomed Pharma, Oslo, Norway). CD14+ monocytes were purified by incubation with anti-CD14-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by sorting with a magnetic device (MiniMacs Separation Unit, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Human monocytic cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (both Sigma-Aldrich, Milan, Italy).

Anti-carbamylated proteins antibody assay
Anti-carbamylated proteins (anti-CarP) were detected by a solid phase ELISA, as previously described [32], using carbamylated fetal calf serum as antigen. The cutoff for anti-CarP antibody ELISA was established as the mean plus three times the s.d. of the healthy controls.

Statistical analysis
Qualitative differences were analysed by the Chi-squared and Fisher’s exact tests. Correlation analysis was carried out by the Spearman test. \( P < 0.05 \) was considered to be statistically significant.

Results
Role of autophagy in generation of carbamylated proteins in human primary fibroblasts
In this study we first evaluated the presence of carbamylated proteins in human fibroblasts following autophagic stimulus, either with 5 \( \mu \)M TNC or with 200 nM rapamycin for different incubation times (Fig. 1A). Western blot analysis, performed using a specific rabbit anti-Carbamyl-Lysine antibody, showed the appearance of numerous bands, corresponding to carbamylated proteins. Densitometric analysis revealed a significant increase of carbamylated proteins as compared with untreated control cells, which was more evident after 4 h of treatment (Fig. 1C).

To better characterize these bands, we focused on the main protein subjected to post-translational modification, that is, vimentin. With this aim, the PVDF membrane was stripped and reprobed with specific anti-vimentin Abs. As expected, the carbamylated band was also stained by these Abs, indicating that autophagic stimuli are able to induce carbamylation of vimentin (Fig. 1B). In samples treated for 4 h, vimentin showed a double band, due to the presence of a cleaved form of the protein (48 kDa), as previously described [9].

Additionally, to confirm these findings, the identity of this band was demonstrated by immunoprecipitation experiments (Fig. 2A). Samples treated with TNC or rapamycin for 20 min or 4 h were immunoprecipitated with anti-vimentin Abs and then subjected to western blot analysis, using anti-Carbamyl-Lysine rabbit polyclonal Ab, which specifically recognizes carbamylated proteins. The analysis revealed that bands, corresponding to vimentin, were stained by the anti-Carbamyl-Lysine Ab in cells treated with TNC or rapamycin. The autophagy induced by TNC or rapamycin treatment was verified by analysis of the expression of LC3-II, a typical marker of autophagic process (Fig. 2B).

Role of autophagy in generation of carbamylated proteins in fibroblast-like synoviocytes from patients with RA
We further analysed the presence of carbamylated proteins following autophagic stimulus, either with TNC or with rapamycin, in fibroblast-like synoviocytes from patients with RA and with OA, a non-inflammatory arthritis (Fig. 3). The results revealed a significant increase of carbamylated proteins in cells from RA patients, as compared with those from OA patients \( (P < 0.01) \). On the basis of previous experiments, RA cells were incubated for 4 h with TNC or rapamycin. Western blot analysis showed the appearance of numerous bands, corresponding to carbamylated proteins (Fig. 3A).
Densitometric analysis revealed a significant increase of carbamylated proteins as compared with untreated cells (Fig. 3B). After stripping of the PVDF membranes, the main carbamylated band was also stained by anti-vimentin Ab, confirming the presence of a double band, as reported below.

Additionally, the identity of this band was demonstrated by immunoprecipitation experiments (Fig. 4A). Samples treated with TNC or rapamycin for 4 h were immunoprecipitated with anti-vimentin Ab and then subjected to western blot analysis, using anti-Carbamyl-Lysine Ab, indicating that the double band corresponding to vimentin was stained by the anti-Carbamyl-Lysine Ab in cells treated with TNC or rapamycin.

Increase in autophagy following treatment with TNC or rapamycin was checked by analysis of LC3-II expression (Fig. 4B). These data indicate that autophagy is able to induce in vitro carbamylation processes, identifying vimentin as the main carbamylated protein.

Evaluation of the correlation between protein carbamylation and autophagy in monocytes of naïve patients with early-active RA

Protein carbamylation, as detected by western blot using the anti-Carbamyl-Lysine Ab, and levels of autophagy, as determined by the autophagic marker LC3-II, were evaluated in 30 samples from naïve patients with early-active RA. The results showed an evident carbamylation in monocytes from naïve patients with early-active RA, as compared with those from control subjects (Fig. 5A).

In order to analyse a possible association between protein carbamylation and levels of autophagy, correlation was evaluated in the 30 naïve patients with early-active RA. The results allowed us to detect an increase in the levels of autophagy in monocytes of those patients who had high levels of monocyte protein carbamylation, albeit with quite high variability among the various patients. Western blot analysis of five representative patients is showed in Fig. 5A. The results are summarized in
Fig. 5 Correlation between monocyte carbamylation levels and LC3-II expression in RA patients

(A) Monocytes from 20 healthy donors and 30 RA patients were analysed, using anti-Carbamyl-Lysine antibody (Ab). Samples from five representative RA patients and one healthy donor are shown. Densitometric analysis of the vimentin band is shown. Autophagy was checked by anti-LC3 polyclonal Ab. Loading control was evaluated using anti-actin mAb.

(B) Histograms represent the mean (S.D.) of densitometric values: carbamylated proteins, ****P < 0.0001 vs control (left panel) and LC3-II/actin ratios, *P < 0.0001 vs control (right panel).

(C) Correlation between carbamylation levels and LC3-II expression in patients with RA. A significant correlation between carbamylation levels (densitometric units of the vimentin band) and LC3-II/actin ratios, revealed by Spearman test, was found (P < 0.0001). Lc3: Microtubule-associated protein 1A/1B-light chain 3.
Fig. 5B as mean (s.d.) of densitometric levels. The densitometric analysis highlighted a significant association between levels of autophagy and anti-Carbamyl-Lysine Ab ($r=0.7654$, $P<0.0001$) (Fig. 5C), as revealed by Spearman’s test.

These findings suggest a link between autophagy and carbamylation processes in naïve patients with early-active RA. The analysis of anti-CarP antibodies revealed the presence of the antibodies in 7 out of the 30 naïve patients with early-active RA (23.3%). Analysing the relationship between clinical and laboratory parameters, and monocyte levels of autophagy and/or carbamylation did not reveal any statistically significant correlation.

Discussion

In this study we demonstrated the role of autophagy in the generation of carbamylated proteins and reported a relationship between the autophagic process and the levels of protein carbamylation in naïve early-active RA patients.

This research starts from previous observations which revealed that autophagy may trigger post-translational modifications of proteins [9]. In particular we observed that, following autophagic stimuli, the hydrolytic conversion of peptidyl-arginin to peptidyl-citrulline is activated by deimination, catalysed by peptidyl arginine deiminases. A significant correlation between levels of protein citrullination and the presence of ACPA in patients with RA had been found [9].

Interestingly, lysine residues undergo a very similar post-translational modification to homocitrulline by carbamylation processes. However, differently from citrullination, carbamylation-dependent homocitrullination is a non-enzymatic mechanism that requires cyanate exposure. Carbamylation may be increased by inflammation, uraemia and smoke inhalation [33]. Recent papers identified IgG specific for carbamylated proteins in patients with RA [34, 35], suggesting that the presence of these antibodies may be useful to predict higher disease activity and may be related to inflammatory biomarkers [36]. In light of these observations, we analysed in vitro the role of autophagy in the activation of protein carbamylation. Our results demonstrated that autophagic cells, treated with either TNC or rapamycin, showed a significant increase of carbamylated proteins, indicating that autophagy is able to induce carbamylation processes in both human fibroblasts and synoviocytes. Immunoblotting and immunoprecipitation experiments identified vimentin as the main carbamylated protein. This finding is not surprising, since a study of early and established RA cohorts reported a strong association between anti-citrullinated vimentin antibodies and anti-CarP Abs [37]. In this context, we demonstrated for the first time the role of autophagy in triggering post-translational modification of proteins, generating both citrullinated [9] and carbamylated vimentin (the present study). Moreover, it is well known that both carbamylated and citrullinated proteins are present in neutrophil extracellular traps [38]. Indeed, a significant cross-road of citrullination and carbamylation processes is also present at the azurophilic granules of neutrophils, where both peptidyl arginine deiminases and myeloperoxidase are active [34]. Thus, we can suggest that both autophagy and NETosis may also play a key role in these processes.

Finally, in this investigation we analysed ex vivo the possible relationship between autophagy and carbamylation levels in early-active naïve RA patients. We focused on early-stage RA patients because of the opportunity to handle patients without pharmacological treatments (such as steroids or DMARDs), which may interfere with our study, since some drugs may interfere with the autphagic mechanism. Our results demonstrated a significant correlation between autophagy and carbamylation levels in mononuclear cells of naïve RA patients, although, at present, analysis of the relationship between clinical and laboratory parameters, and monocyte levels of autophagy and/or carbamylation, did not reveal any statistically significant correlation.

It could be considered an early trigger for the appearance of anti-CarP Ab [39, 40], which in turn precede the onset of RA [34]. Indeed, in this study we observed the presence of anti-CarP antibodies only in 23.3% of early-active RA patients, supporting the view that carbamylation represents an early trigger, which precedes the appearance of the antibodies. A follow-up study is in progress to evaluate whether carbamylation may represent an useful tool for evaluating the future risk of disease progression.

In conclusion, these findings support the view that post-translational processing of proteins in autophagy may generate carbamylated proteins recognized by the immune system in early-active RA [41]. These observations introduce a new pathogenetic mechanism of disease, knowledge of the molecular aspects of which may contribute to more accurate monitoring of patients.

Acknowledgements

We thank Dr Tania Colasanti for her helpful assistance.

Funding: This work was supported by a grant from Sapienza University project 2016.

Disclosure statement: The authors have declared no conflicts of interest.

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