Homocysteine enhances cytokine production in cultured synoviocytes from rheumatoid arthritis patients

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Abstract

Objective

Hyperhomocysteinemia is commonly observed in Rheumatoid Arthritis (RA) patients, thus putatively accounting in part for the high rate of cardiovascular events in these subjects. Homocysteine (Hcy) is known to exert a pro-inflammatory effect putatively contributing to the progression of atherosclerotic lesions by cytokine production from several vascular cell-types.

In order to evaluate the possibility that Hcy may play a direct pro-inflammatory activity also in the joints of RA patients, we investigated: (i) the joint concentration of Hcy, and (ii) the effect of Hcy on cytokine production by unstimulated and IL-1β-stimulated human RA cultured synoviocytes.

Methods

In 5 RA and 5 controls subjects, Hcy was measured in the blood and knee synovial fluid, and specimens of synovial tissue were taken to obtain cell cultures. Cultures were incubated with Hcy (10-100 μmol/l) ± IL-1β, and IL-6 and IL-8 concentrations were evaluated in the supernatants (ELISA) together with the activation of nuclear factor-kB (NF-kB) (immunocytochemistry).

Results

Hcy was present in synovial fluids, with a mean concentration significantly higher in RA patients than in controls (9.0 ± 1.1 vs 5.9 ± 1.2 μmol/l). Hcy enhanced IL-6 and IL-8 production in RA synoviocytes only (up to 35%). Moreover, Hcy produced a clear-cut activation of NF-kB in rheumatoid cells only.

Conclusion

Hcy enhances IL-1-dependent cytokine production by rheumatoid synoviocytes at a concentration measurable in RA joints in vivo. *Thus, in RA patients, Hcy may not only represent an important risk factor for the progression of cardiovascular diseases, but it may also contribute to the joint damage.*

Key words

Homocysteine, rheumatoid arthritis, interleukin-6, interleukin-8, NF-kB, inflammation, synoviocytes.

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Introduction

Mild hyperhomocysteinemia, as defined on the basis of plasma concentration ranging between 16-30 μmol/l, is an independent risk factor for coronary artery disease, cerebral and peripheral vascular disease (1-3), and for deep-vein thrombosis in the general population (4). Homocysteine-dependent vascular damage would be related to direct endothelial toxicity and indirect mechanisms, such as the induction of a prothrombotic state mediated by the effects on coagulation factors and platelets, and atherogenic modification of LDL. Moreover, recent studies demonstrated that homocysteine (Hcy) is also provided with either immunomodulating and pro-inflammatory activities. More in detail, Hcy enhances the production of molecules such as IL-6, IL-8, and monocyte chemoattractant protein-1 by monocyte-macrophages and endothelial cells, and nitric oxide, matrix metalloproteinase-9 and vascular cell adhesion molecule-1 (VCAM-1) by vascular smooth muscle cells (5-9), thereby suggesting a possible additional role for Hcy in the inflammatory process supporting atherogenesis. These effects would be referred to an enhanced gene expression related to Hcy-induced activation of the nuclear factor kB (NF-kB), mediated by the production of superoxide anion (8-10).

High plasma levels of Hcy are commonly observed in patients with Rheumatoid Arthritis (RA), thus putatively accounting, at least in part, for the high rate of mortality from cardiovascular events in these subjects (11-18). The exact mechanisms leading to hyperhomocysteinemia in RA have not been clarified as yet. However, pharmacological therapy (methotrexate and sulphasalazine) is thought to play relevant effects on Hcy-related biochemical pathways, both reducing vitamins absorption (gastrointestinal toxicity), and interfering with folate metabolism (11, 14, 17). Moreover, recent data suggest that also the immune activation which characterizes RA could be involved in the development of hyperhomocysteinemia, through an enhanced demand and/or accelerated catabolism of vitamins (folate, B12, B6) playing a key role in the sulphured amino-acids metabolism (19-22).

So far, the possible role of the above mentioned pro-inflammatory activity of Hcy in the progression of RA has never been studied.

Synoviocytes are the cells mainly involved in the development of cartilage damage in chronic inflammatory joint diseases, particularly RA (23). These cells play a relevant pathogenetic role by producing several cytokines, including IL-6 and IL-8, when triggered by IL-1β (24-27) which activates NF-kB, leading to the transcription of the genes encoding for such cytokines (28, 29).

In order to evaluate the possible occurrence of a direct pro-inflammatory activity of homocysteine in the progression of joint damage in RA patients, we investigated: (i) the intra-articular concentration of Hcy in RA patients, and (ii) the effect of different concentrations of Hcy on cytokine production by human cultured synoviocytes from patients with RA, following stimulation with IL-1β and in resting conditions.

Materials and methods

Patient selection

Five patients with active RA were enrolled whose demography is depicted in Table I. All patients met the American College of Rheumatology 1987 revised criteria for rheumatoid arthritis (30). Active RA was defined as a European League Against Rheumatism Disease Activity Score in 28 joints (DAS28) of >3.2 (31). All the patients had an inflammatory knee joint involvement. Specimens of synovial tissue were obtained during diagnostic procedures for RA patients by needle biopsy. Before biopsy, synovial fluid was aspirated, and wet analysis by ordinary and polarised light microscopy was performed to confirm the inflammatory pattern of the effusion and to exclude the coexistence of other possible inflammatory processes. Patients were considered eligible for the study if synovial fluid leukocyte count was comprised between 2,000 and 10,000 cells/mm^3. The control group (demography in Table I) was represented by five sex- and age-matched pa-

tients with osteoarthritis (OA) of the knee who underwent knee joint prosthesis; synovial fluid and synovial tissue specimens were obtained soon before and during joint replacement surgery, respectively. Also in this group, analysis of the synovial fluid by ordinary and polarised light microscopy was performed.

Exclusion criteria were current infections or neoplasms. Oral informed consent was obtained in accordance with the Principles of the Declaration of Helsinki.

All patients were under steroid treatment (mean daily dose < 8 mg of prednisone-equivalent) and/or nonsteroidal anti-inflammatory drugs. At the time of the study entry, patients were neither under disease modifying anti-rheumatic drugs, nor under TNFα-blocking therapy. More in detail, DMARDs treatment was discontinued at least 1 month before sample collection, for either inefficacy (2 patients), or occurrence of side effects (3 patients).

The day of synovial sampling, blood was withdrawn from an antecubital vein and anticoagulated with sodium-citrate for the measurement of Hcy plasma levels.

Homocysteine measurement in blood and synovial fluid

Total Hcy was measured in plasma and synovial fluid using tri-n-butylphosphine and 7-fluoro-benzo-2oxa-1,3-diazole-4-sulphonate. The derivatives were separated by reversed-phase high-performance liquid chromatography. In our laboratory and in the literature the normal control values for Hcy level in plasma range from 5 to 15 μmol/l; no data are available in the literature about the level of Hcy in the synovial fluid.

Synoviocyte cultures

Immediately after sampling, 2 mm^3 of synovial tissue from patients in both groups were removed in aseptic conditions and minced in into 1 mm^3 pieces. Synovial fragments were washed in saline solution (PBS, containing 200 U/ml penicillin and 200 μg/ml streptomycin) (Sigma-Aldrich, Milan, Italy). Synovial tissue was then digested by clostridial collagenase (Sigma-Aldrich, Milan, Italy) 1 mg/ml in PBS or in free serum Dulbecco's Mod Eagle medium (DMEM; Invitrogen, Milan, Italy) containing the same concentration of antibiotics.

Collagenase digestion was carried out at 37°C for 24 hours with moderate stirring. The solution was then washed in saline solution and centrifuged for 10 minutes at 700g. Cell suspensions were submitted to trypan blue viable stain (usually 90-95% of the cells recovered were alive).

Cell suspensions obtained were plated out in 2 ml of DMEM supplemented with L-glutamine (Sigma-Aldrich, Milan, Italy) (2mM), foetal calf serum (FCS; Sigma-Aldrich, Milan, Italy) (10%), penicillin (200U/ml) and streptomycin (200 μg/ml) in 35 mm/tissue culture dish (Sarstedt, Nümbrecht, Deutschland) (35 x 10 mm style) in humidified atmosphere containing 5% CO_2, and were grown to confluence with medium changes every other day. After confluence, cells were plated out in 100 mm/tissue culture dish (100 x 20 mm style), and grown to the new confluence two more times. We obtained 4 culture dishes (100 x 20 mm style), with a final concentration of 1.5 x 10^6 cells per dish.

The number of passages was carefully monitored in order to keep the cells well differentiated.

Synovial fibroblasts were plated out in 400 μl of complete medium (DMEM without red phenol supplemented with penicillin 200 U/ml, streptomycin 200 μg/ml, and 2.5% FCS) in 48-well tissue culture plate (1x10^5 cells/well) and allowed to attach for 24 hours.

Culture stimulation

Culture medium was replaced with fresh complete medium containing DL-Hcy (10,20,50,100 μmol/l) (Sigma-Aldrich, Milan, Italy) with human recombinant IL-1β (0.1 ng/ml; Boehringer-Mannheim, Germany). The concentration of IL-1β employed is that commonly found in the synovial fluid of RA patients (32). After 8 and 24 hours of incubation, the supernatant was collected and stored at –20°C.

The effect of DL-Hcy (10,20,50,100 μmol/l) alone was also evaluated in comparison with untreated cells, in the same experimental conditions after a 24h incubation. The effect of the vehicle PBS was also evaluated as a blank.

Cytokine assay

The concentration of immunoreactive IL-6 and IL-8 in culture supernatants was measured by a colorimetric sandwich ELISA kit (Euroclone Lugano, Switzerland). The concentration of the cytokines was expressed as ng/ml. Supernatants from each well were tested for cytokine assay in duplicate.

Evaluation of NF-kB activation

Cultured synoviocytes from two RA and two OA patients were immunostained for the NF-kB p65 subunit, i.e. the active form of the NF-kB, in basal condition, after 50 μmol/l Hcy incubation and after IL-1β incubation. Stimulated synoviocytes from patients were detached from culture plate by trypsine treatment, and then resuspended in PBS to a final concentration of 300 cells/-mm^3. 100 μl of the cell suspension were centrifuged onto poly-L-lysine coated slides, air dried and 10 min fixed in precooled acetone. Slides were incubated 1 hour at 4 °C with the anti-human mouse monoclonal IgG NF-kB p65 (Santa-Cruz Biotechnology California, USA) diluted 1:400 in PBS. Immuno-reactions were then developed by using Vectastain ABC Kit (Vector Laboratories, Burlingame, CA USA) according to the data sheet specifications and stained by diaminobenzidine solution (Sigma-Aldrich, Milan, Italy).

Cell viability

Cell viability was evaluated after cell incubation with an increasing concentration of Hcy (10-100 μmol/l) in the culture medium for 48 hours by methylthiazoletetrazolium (MTT; Sigma -Aldrich, Milan, Italy) and trypan-blue exclusion test (33). The control culture was obtained by incubating cells with culture medium for 24 hours. Results were expressed as mean ± SD.

Statistical analysis.

The difference in plasma and synovial fluid Hcy concentration between RA patients and controls, was estimated by

a two-tailed unpaired Student's *t* test. Statistical evaluation of the effect of Hcy treatment ± IL-1β on RA- and control-derived synoviocytes was performed by the one-way analysis of variance for repeated measurements (RM-ANOVA) for normally distributed data, and by Friedman repeated measurements analysis of variance on ranks (RM-ANOVA on Ranks) for non normally distributed data. Then a "post-hoc" test (Student-Neuman-Keul's test for multiple comparisons) was employed to compare the effects of the different treatments. The level of significance was set at $P < 0.05$.

Table I. Demographic characteristics of patients. Data are expressed as mean ± SD [range].

	OA	RA
Age (years)	62 ± 5 (58-71)	56 ± 8 (48-69)
Sex (n women/n men)	3/2	3/2
Disease duration (months)	72 ± 69 (10-110)	74 ± 38 (26-133)
DAS 28	-	4.1 ± 0.5 (3.6-5)
Synovial fluid leukocyte count (cell/mm^3)	680 ± 430 (200-1300)	5500 ± 3200 (2000-9800)
Synovial fluid volume (ml)	22 ± 8 (15/35)	23 ± 6 (15/30)
ESR (mm/1st hour)	17 ± 3 (14/23)	51.9 ± 22 (35/81)

Results

Synovial fluid analysis

Synovial fluid from osteoarthritis patients showed a typical "non-inflammatory pattern", characterized by a transparent yellow coloured, viscous macroscopic appearance, whereas synovial fluid from RA patients showed an inflammatory pattern with reduction in viscosity and transparency. No crystals were detected by ordinary and polarised light microscopy in all the samples studied. The mean volume aspirated and the leukocyte count are reported in Table I.

Homocysteine level in blood and synovial fluid

Mean Hcy plasma level seemed slightly higher in RA patients than in controls, but the difference did not reach statistical significance (16.5 ± 2.1 vs 14.8 ± 2.4 μmol/l); on the contrary, mean Hcy concentration in the synovial fluid was significantly higher in patients with RA than in the controls (9.0 ± 1.1 vs 5.9 ± 1.2 μmol/l; $p = 0.001$). As a matter of fact, synovial/plasma Hcy ratio was higher in the RA than in the control group (0.54 vs 0.40) (Fig.1).

On the basis of these findings, we stimulated synoviocyte cultures also with an Hcy concentration of 10 μmol/l to mimic the pathophysiological conditions really operating *in vivo* in RA patients.

Cytokine production

Co-incubation of Hcy and IL-1β was able to time- and dose-dependently enhance IL-6 production in cultured synoviocytes from RA patients (Table II). Conversely, no effect was observed on cultures from controls (Table II). The extent of IL-6 production was greater after 24 h (Table II) than it was after 8 h (Table II) cell incubation with Hcy+IL-1β. The maximal stimulating effect was reached with 20-50 μmol/l Hcy, after 8 and 24 h incubation. Interestingly, the increase in cytokine levels after incubation with 100 μmol/l Hcy was less relevant than that observed with 50 μmol/l Hcy (Tab. II). Indeed, it is remarkable that also 10 μmol/l Hcy was able to induce a significant cytokine increase after 24 h incubation (Table II).

A similar behaviour was observed in the production of IL-8 after 8 h (Table III) and 24 h (Table III) incubation with Hcy+IL-1β.

The extent of the maximal stimulating effect of the co-incubation with Hcy+ IL-1β with respect to IL-1β alone was over 30 percent for both cytokines (+32% for IL-6 and +36% for IL-8).

Synoviocytes produce a low amount of IL-6 and IL-8 (Table IV) in resting conditions, i.e., without co-incubation with IL-1β. Synoviocytes from RA patients produce slightly higher amounts of the cytokines than cells from controls (Table IV). In these experimental conditions, the addition of Hcy produced a significant stimulating effect on cytokine production only in synoviocytes from RA patients (Table IV).

NF-kB activation

On immunohistochemistry preparations, positive reactions for the NF-kB p-65 subunit were observed in all the slides from RA patients. Interestingly, a mild immunoreaction was present also in cytospin preparations from unstimulated rheumatoid synoviocytes, as an expression of a constitutive activation

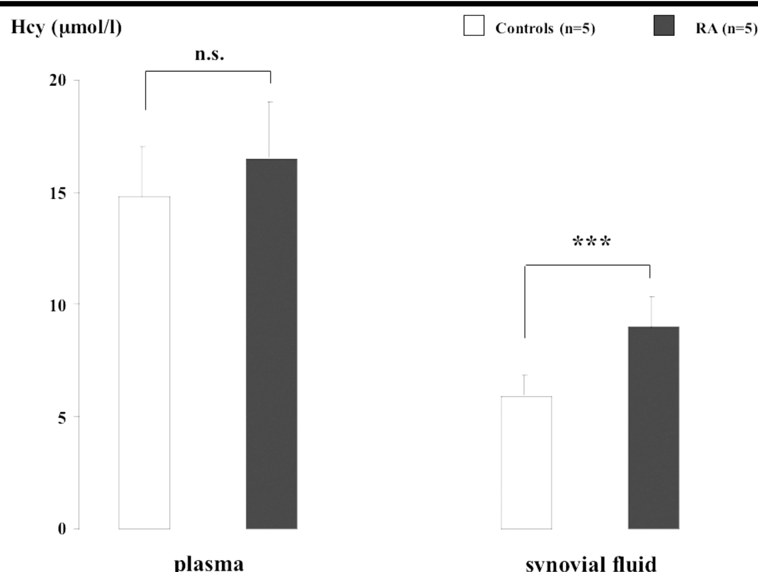

Fig. 1. Levels of Hcy in plasma and synovial fluid of rheumatoid arthritis (RA, n = 5) and osteoarthitis (controls; n = 5) patients. Student's "t" test for unpaired data (RA vs controls). *** = $p < 0.001$.

Table II. Effect of 10-100 μmol/L Hcy on 0.1 ng/ml IL1β-induced IL-6 production by cultured synoviocytes from rheumatoid arthritis (RA, n = 5) and osteoarthitis (controls; n = 5) patients after 8 h, and 24 h incubation. Repeated measurements oneway analysis of variance (RM ANOVA): § = $p < 0.05$, §§§ = $p < 0.001$. Student-Neuman-Keuls' test (Hcy-treated vs Hcy untreated IL1β-incubated cells from RA patients): * = $p < 0.05$. Data are expressed as Median (range).

IL-6 (ng/ml)		IL-1	IL1+Hcy10	IL1+Hcy20	IL1+Hcy50	IL1+Hcy100	RM ANOVA
8h	*C*	6.94 (5.19-10.52)	7.37 (4.20-11.86)	7.54 (4.39-11.71)	7.92 (4.09-12.29)	7.56 (5.84-11.01)	n.s.
	RA	18.45 (12.57-24.53)	20.46 (13.40-28.94)	23.60* (16.94-30-51)	24.54* (17.04-33.71)	23.92* (17.18-31.16)	§
24h	*C*	44.64 (29.79-55.54)	43.80 (26.05-57.90)	46.95 (22.43-59.85)	45.25 (24.25-54-30)	46.01 (28.18-56.90)	n.s.
	RA	80.17 (51.59-99.71)	92.30* (53.93-124.28)	99.37* (55.05-140.74)	102.72* (59.75-138.45)	96.73* (57.89-127.51)	§§§

Table III. Effect of 10-100 μmol/L Hcy on 0.1 ng/ml IL-1β-induced IL-8 production by cultured synoviocytes from rheumatoid arthritis (RA, n = 5) and osteoarthitis (controls; n = 5) patients after 8 h, and 24 h incubation. Repeated measurements oneway analysis of variance (RM ANOVA): §§ = $p < 0.01$. Student-Neuman-Keuls' test (Hcy-treated vs Hcy untreated IL1β-incubated cells from RA patients): * = $p < 0.05$. Data are expressed as Median (range).

IL-8 (ng/ml)		IL-1	IL1+Hcy10	IL1+Hcy20	IL1+Hcy50	IL1+Hcy100	RM ANOVA
8h	*C*	5.24 (3.56-7.54)	5.73 (3.61-7.73)	5.64 (3.79-7.29)	6.18 (3.74-8.84)	6.02 (3.21-7.89)	n.s.
	RA	9.72 (6.55-13.75)	10.61 (7.57-15.32)	13.67* (8.99-19.30)	13.48* (8.88-19.42)	11.96* (9.68-17.21)	§§
24h	*C*	28.18 (21.47-40.35)	29.56 (22.10-41.30)	29.84 (21.59-40.05)	29.06 (21.80-40.36)	28.24 (20.08-42.30)	n.s.
	RA	34.62 (24.08-44.99)	38.10* (26.02-51.87)	40.01* (28.01-53.58)	40.64* (30.01-53.23)	38.58* (28.81-49.25)	§§

Table IV. Effect of 10-100 μmol/L Hcy on spontaneous IL-6 and IL-8 production by cultured synoviocytes from rheumatoid arthritis (RA, n = 5) and osteoarthitis (controls; n = 5) patients after 24 h incubation. Repeated measurements oneway analysis of variance (RM ANOVA): § = $p < 0.05$. Student-Neuman-Keuls' test (Hcy-treated vs Hcy untreated cells from RA patients): n.s. Data are expressed as Median (range).

		Baseline	Hcy10	Hcy20	Hcy50	Hcy100	RM ANOVA
IL-6 (ng/ml)	*C*	1.34 (0.80-1.92)	1.37 (1.06-1.72)	1.53 (0.84-2.13)	1.49 (0.83-2.17)	1.29 (0.98-1.61)	n.s.
	RA	1.75 (1.28-2.74)	2.62 (1.59-3.99)	2.90 (2.11-4.12)	2.78 (1.71-4.21)	2.06 (1.34-3.59)	§
IL-8 (ng/ml)	*C*	0.49 (0.37-0.65)	0.48 (0.39-0.59)	0.52 (0.30-0.67)	0.51 (0.39-0.60)	0.48 (0.40-0.56)	n.s.
	RA	0.87 (0.55-1.09)	1.02 (0.72-1.25)	1.05 (0.78-1.28)	1.07 (0.65-1.49)	0.97 (0.69-1.20)	§

of NF-kB. This condition markedly increased after Hcy incubation. However, as expected, IL-1β stimulation led to the most pronounced immunoreaction, since almost all the synoviocytes showed a strong immunostaining (Fig. 2). Conversely, in the slides from OA patients no immunoreactivity was observed in basal conditions; a mild reaction was detected after Hcy incubation. However, also in this case, a marked immunostaining was detected after stimulation with IL-1 β (Fig. 2).

Cell viability

Hcy concentrations in the culture medium as high as 10, 20, and 50 μmol/l were associated with a cell viability > 90 %; at the 100 μmol/l Hcy concentration, cell viability was 83 ± 4 %.

Discussion

Mild hyperhomocysteinemia is an important risk factor for cardiovascular diseases and it is putatively involved in the development of the accelerated atherosclerosis associated with RA. Hcy promotes vascular damage also exerting a pro-inflammatory effect mediated by cytokine production from several cell-types implicated in the atherosclerotic process.

We investigated the possibility that in RA patients Hcy may represent not only a cardiovascular risk factor, but also an active mediator of the inflammatory process involved in the progression of joint damage.

In this study, for the first time, we provide evidence that detectable amounts of Hcy are present in synovial fluids from RA and OA. Interestingly, we found that Hcy concentration in the synovial fluid from RA patients was significantly higher than that from control subjects with OA. In RA patients higher synovial levels of Hcy suggest that the nature of the joint disease may influence Hcy intra-articular levels. A possible explanation for the phenomenon may consist in the increase in the blood in-flow and permeability in the synovial tissue due to the inflammatory condition of the joint in RA. However, we cannot rule out the possibility that an enhanced synthesis or/and deficient catabolism of Hcy take place intra-articularly in RA patients. Starting from these *ex-vivo* observations, we evaluated whether the higher levels of Hcy could influence the development

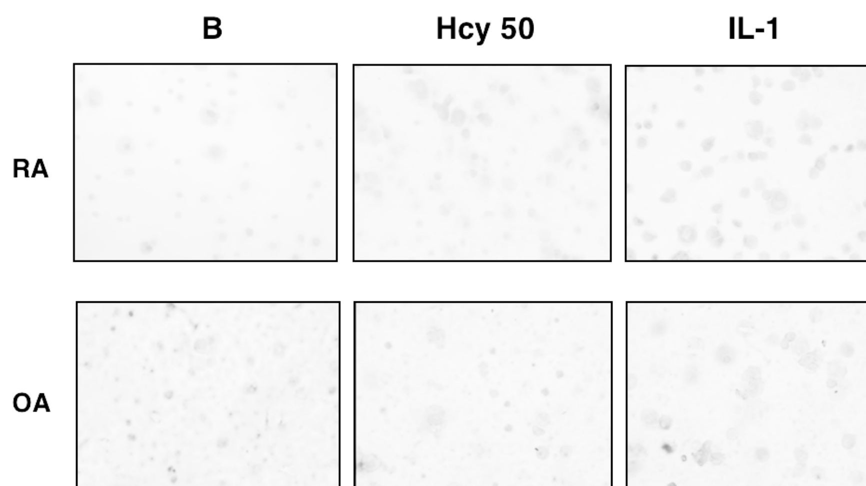

Fig. 2. Cytospin preparations of synovial cells from RA and OA patients immunostained by anti-human mouse monoclonal IgG NF-kB p65. B: basal conditions, i.e. unstimulated cells; Hcy 50: with 50 μmol/l Hcy incubation; IL-1: with IL-1β (0.1 ng/ml) stimulation.

of the proinflammatory loop leading to rheumatoid joint damage. In accordance with our hypothesis we showed that Hcy significantly enhances IL-6 and IL-8 production by synoviocytes from RA patients, particularly in the presence of co-stimulation with IL-1β. The effect is described by a biphasic curve: an initial progressive increase in IL-6 and IL-8 levels for Hcy concentrations from 10 to 50 μmol/l, followed by a slight decline for Hcy concentration of 100 μmol/l. Conversely, no appraisable effect of Hcy on cytokine production from OA-derived cultured synoviocytes was detected. These findings suggest that RA synoviocytes bear peculiar features, maybe related to a relevant and persistent inflammatory stimulation and/or genetic predisposition, with a non-specific pro-inflammatory attitude and a higher sensitivity to Hcy stimulation. It seems remarkable that the concomitant increase in both IL-6 and IL-8 induced by Hcy in our study, may putatively exert a cumulative effect on rheumatoid inflammatory process. In fact, either IL-6 and IL-8 are critically involved in the development of RA, and levels of both cytokines correlate with the clinical behaviour of the disease (34, 35).

A solid body of evidence from the literature demonstrates that NF-kB is constitutively activated in RA synovial tissue (29), and Au-Yeung and Co. (10) showed that in human endothelial cells Hcy displays its proinflammatory activity via NF-kB activation. These concepts support the hypothesis that Hcy-induced cytokine production in synovial cells is mediated via NF-kB pathway activation, and that NF-kB constitutive activation could account for the particular sensitivity to Hcy of RA synoviocytes. Our data seem to be consistent with this point of view. In fact, in RA synoviocytes, but not in controls, a slight immunoreactivity for activated subunit p-65 NF-kB was detectable also in basal conditions; immunoreactivity of the cells markedly increased after incubation with Hcy only in the cytospins of RA patients.

Georganos and Co. (29) demonstrated the dominant role for NF-kB in the regulation of IL-6, IL-8 expression in rheumatoid type–B synoviocytes; along with this observation, and in the light of the above mentioned experimental results, we postulate that Hcy may promote an increased expression of IL-6 and IL-8 mRNA leading to cytokine neosynthesis.

At this moment, the observation that a higher Hcy concentration (> 50 μmol/l) exerts less potent effect on cytokine production has not a clear explanation. In fact, the data on cell viability suggest that some toxic effects occur at that Hcy concentration, responsible for a synoviocyte loss of about 17%. However, a dual concentration-dependent effect of Hcy on cytokine production cannot be ruled out, with a stimulating activity at low-medium concentration, and an inhibitory activity at high concentration.

Our data seems to suggest that, in patients affected with RA, Hcy may represent not only an important risk factor for the development of cardiovascular disease, but also a factor actively involved in the progression of joint damage. This conclusion is particularly supported by the observation that in our study the stimulating effect on cytokine production occurs for Hcy and IL-1β concentrations measured in RA joints *in vivo.*

As a consequence, the preservation of normal plasma level of Hcy in patients with RA may become a crucial goal in order to affect not only the accelerate systemic cardiovascular involvement, but also the immuno-inflammatory load sustaining the destroying effect of the disease on the joints. Indeed, many studies clearly demonstrated the effectiveness of vitamin supplementation (mainly folate) in lowering Hcy plasma level in hyperhomocysteinemic subjects (36, 37), also affected with RA under methotrexate treatment, at least in adults (18, 38, 39). Folate supplementation is effective in reduce Hcy level also in patients not presenting evidence of vitamin deficiency (40). Conversely, no data are presently available on the possible reducing effect of such vitamins on synovial Hcy concentration. Since the present study seems to suggest a relationship between plasma and synovial Hcy levels, it seems conceivable that folate treatment may influence also homocysteine concentration in the joints.

On this basis, a study aiming at evaluating the possible concomitant modifications of Hcy level in the blood and in the synovial fluid in RA patients after the onset of folate therapy is now in progress.

The expected results of this study and the data obtained from the present work may strengthen the rationale for the addition of folate to the standard therapeutic regimen in all RA patients with hyperhomocysteinemia. Such a consideration seems to be of particular relevance in European countries; in fact bread and cereals are universally supplemented with folate in North Ameri-

ca, thus leading to lower mean plasma levels of Hcy in the whole population.

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