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Differential effects of osteopontin on the cytotoxic activity of macrophages from young and old mice

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SUMMARY

Osteopontin (OPN) is a secreted phosphoprotein found in body fluids (e.g. plasma, urine, milk) and in mineralized tissues. Its expression is increased in many transformed cells and in normal cells exposed to various cytokines. When stimulated with the inflammatory mediators lipopolysaccharide and interferon-γ, mouse macrophages secrete nitric oxide (NO) as a cytotoxic agent effective against microbial invaders and tumour cells. This report documents (1) that thioglycollate-elicited peritoneal macrophages, activated with the inflammatory mediators, produced less NO and exhibited reduced cytotoxicity towards target cells when they were obtained from old animals than when they were obtained from young animals; and (2) that OPN was able to inhibit both the induced NO synthesis and cytotoxicity, but more effectively in macrophages from the young animals than those from the old animals. This may be due to the observed higher level of OPN expression in macrophages from old animals.

INTRODUCTION

The immune system becomes progressively dysfunctional with age. There is a gradual loss of the proliferative capacity of the cells and an accompanying change in the relative proportions of the different cell types that parallels changing patterns of cytokine expression. The ability of the cells to be activated by extracellular signals is lessened, and there is a decline in their capacity to react to foreign antigens, possibly coinciding with an age-related appearance of autoantibodies.[1–5] In old animals there is an increase, compared with young animals, in the formation of free radicals by mitochondria and consequent injury to cell constituents; this has been suggested to contribute causally to several diseases associated with ageing.[6,7]

Proximal tubule epithelial (PTE) cells from the kidneys of adults and old people possess higher levels of constitutive nitric oxide synthase (cNOS) mRNA than do those of the very young, and in the 'old' PTE cells there is an increased production of nitric oxide (NO) during hypoxia/reoxygenation and increased cell death in comparison to cells from kidneys of the very young.[8] Osteopontin (OPN) expression in vascular smooth muscle cells of rat aorta and carotid artery and the mouse kidney also increase with the age of the animal.[9,10] It may be that age-related changes in OPN and NO production contribute to alterations in the susceptibility of cells to oxidants during ageing.

OPN is found in a variety of tissues and body fluids, and is thought to have a role in normal and pathological calcification processes.[11] It contains several conserved motifs, including a thrombin cleavage site and the integrin adhesion sequence Gly–Arg–Gly–Asp–Ser (GRGDS), which facilitates cell attachment and interacts with the integrin $\alpha_v\beta_3$. The low level 'constitutive' expression of OPN by fibroblasts, resident macrophages, macrophage cell lines, T lymphocytes, and splenic natural killer (NK) cells can be enhanced by a variety of stimuli.[12–16] Purified OPN binds specifically to mouse macrophages and other cells, through an RGD-containing domain, and subcutaneous administration of OPN generates a macrophage infiltrate.[15]

The OPN concentration in normal human plasma (30–40 ng/ml; J. Harris, personal communication) is significantly increased in the plasma of patients with septic shock or metastatic disease (50–200 ng/ml).[17] Most tumour cells, particularly *ras*-transformed cells, produce at least 10-fold or more OPN than do their normal or untransformed counterparts.[18,19] Down-regulation of OPN expression in *ras*-transformed cells with antisense RNA reduces their tumourigenicity and metastatic ability.[20] Because macrophage NO production is both tumouristatic and tumouricidal, with a decrease in macrophage NO production being more permissive for tumour growth, it is relevant that OPN inhibits NO production induced by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) in primary human kidney cell cultures[21] and in the mouse macrophage-like RAW264·7 cells (E. E. Rollo, D. L.. Laskin and D. T. Denhardt, manuscript submitted for publication). OPN also decreases the

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Abbreviations: FBS, fetal bovine serum; IFN, interferon; IPTG, isopropyl β-D-thiogalactopyranoside; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; NOS, nitric oxide synthase; OPN, osteopontin; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PTE, proximal tubule epithelial.

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cytotoxicity of macrophages towards tumour cells (E. E. Rollo *et al.*, manuscript submitted for publication; and this work).[22] We report here that in comparison to macrophages elicited from young mice, macrophages elicited from old mice are less responsive to the inflammatory mediators and are less susceptible to an inhibitory action of OPN, as assessed both by NO release and by their ability to kill tumour cells.

MATERIALS AND METHODS

Cytokines and reagents

Mouse IFN-γ (400 U/μl) was the kind gift of Dr S. Pestka (Robert Wood Johnson Medical School, Piscataway, NJ). LPS was from *Escherichia coli* Serotype 0128:B12 (Sigma Chemical Company, St Louis, MO). Anti-2arC, an affinity-purified antiserum against the 80-amino acid C-terminal portion of mouse OPN-β-galactosidase fusion protein synthesized in *E. coli*, was generated in this laboratory.[23] OPN was purified as described by E. E. Rollo *et al.* (manuscript submitted for publication). All tissue culture media were free of detectable endotoxin, as determined by assay with Limulus amoebocyte lysate (ICN, Costa Mesa, CA), at a sensitivity of 0·06–0·10 ng/ml.

Animals

Young (7–9 weeks) and old male (22–26 months) C57BL/6 J mice were obtained from in-house matings, and were the kind gift of X.-R. An (Rutgers University, Piscataway, NJ). Peritoneal cells were not used in the study if the donor mice appeared ill or if any anatomic abnormalities were noted at the time of cell harvest.

Mammalian cell culture

Thioglycollate-elicited mouse macrophages, isolated as described below, were maintained in RPMI-1640 supplemented with 2 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS). Mouse mastocytoma cell line P815 (ATCC TIB 64; Rockville, MD) was grown in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 10% FBS, 50 μg/ml penicillin and 50 μg/ml streptomycin at 37° in an atmosphere of 5% CO_2, and subcultured by scraping when 80% confluent.

Preparation of thioglycollate-elicited macrophages

A 4% sterile solution of Brewer's thioglycollate broth (Difco, Detroit, MI) was injected intraperitoneally (i.p.) into mice with a 26-gauge needle; 1·5 ml were injected into mice younger than 2 months, and 3 ml were injected into mice 2 months and older. Four days after injection, the peritoneal exudate cells (PEC) were harvested. The mice were killed by cervical dislocation, and inflammatory macrophages were harvested by peritoneal lavage: 10 ml of sterile phosphate-buffered saline (PBS), pH 7·4, was injected intraperitoneally into the mice, the abdomens were massaged for 30 s, and the fluid withdrawn. Up to 8 ml of fluid was recoverable. The cells were then washed once with PBS, resuspended in medium, counted, and plated using the following formula[24] to obtain $2{\cdot}5 \times 10^6$ macrophages/ml:

$$(x \text{ PEC/ml}) \times (90\% \text{ macrophages}) \times (0{\cdot}5 \text{ adherent}) = 2{\cdot}5 \times 10^6 \text{ macrophages/ml.}$$

After allowing the peritoneal macrophages to adhere overnight, non-adherent cells were removed by washing three times with PBS. The cell populations were confirmed consistently as 50% adherent by counting, plating, removing adherent cells and recounting; 90–95% of the adherent cells were macrophages as judged by staining with Wright's Stain (Sigma) and by the non-specific esterase reaction (Sigma). This procedure yielded $3–4 \times 10^6$ macrophages/mouse.

Preparation of radiolabelled probes for Northern blot analysis

The cDNAs used to make probes for the Northern blot included β-actin,[19] the *Hin*dII fragment of mouse OPN[23] and the *Nco*I fragment of iNOS,[25] each random-primer labelled with 25 μCi of [α^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml; Amersham Corp., Arlington Heights, IL) using the Oligolabelling Kit from Pharmacia Inc. (Piscataway, NJ) according to the manufacturer's protocol.

RNA isolation and Northern blot analysis

RNA was isolated using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instructions and was analysed on a 1·5% agarose gel containing formaldehyde. After electrophoresis, the RNA gel was blotted to a GeneScreen Plus membrane (DuPont-NEN, Boston, MA), which was then hybridized according to the protocol outlined in the GeneScreen Plus manual. After hybridization, blots were washed and autoradiographed. β-actin and iNOS probes were hybridized together, the blots stripped by boiling the membrane for 30 min in 0·1× SSC containing 1·0% sodium dodecyl sulphate (SDS) and rehybridized with the OPN probe. Hybridization was quantified by scanning autoradiograms using an LKB UltroScan XL densitometer (Pharmacia/LKB, Piscataway, NJ).

Assay for NO synthesis

Synthesis of NO was estimated by assaying culture supernatants for the presence of nitrite, a stable reaction product of NO, generated by macrophages alone or in the presence of tumour cell targets.[26] Fifty microlitres of each cell-free culture supernatant were incubated with 50 μl of Griess reagent (0·5% sulphanilamide, 0·05% N-[1-naphthyl]ethylenediamine dihydrochloride in 2·5% phosphoric acid) in a 96-well flat-bottomed tissue culture plate for 10 min at room temperature in the dark. The optical densities of the assay samples were then measured at 540 nm with a Bio-Tek EL310 Microplate Autoreader (Bio-Tek Instruments, Inc., Winooski, VT), using a solution of phenol red-free DMEM + Griess reagent as a blank.

Assay for macrophage-mediated tumouricidal activity

Cytotoxicity of macrophages was assayed by a modification of the procedure of Lorsbach *et al.*[27] Thioglycollate-elicited macrophages isolated as described above were plated in phenol red-free RPMI-1640 supplemented with 5% heat-inactivated FBS in 96-well flat-bottomed tissue culture plates (Corning Inc., Corning, NY) at the densities indicated in the figures, and incubated for 2 hr at 37° to allow them to adhere. The contents of the wells were then gently aspirated, 100 μl of fresh medium ± 100 ng/ml LPS and 100 U/ml or 1000 U/ml IFN-γ (as indicated) ± OPN added to each well, and the cells incubated for 8 hr at 37°. Approximately 4 hr after stimulation of the macrophages, P815 mastocytoma target cells were

labelled with 250 μCi of sodium [^{51}Cr]chromate in aqueous solution (CJS.1V; Amersham Corp.) for 1 hr in a sterile 35-mm bacterial Petri dish with occasional swirling. The labelled target cells were then washed, resuspended in medium, and allowed to 'leak' for at least 1 hr, after which they were washed again, resuspended in medium, counted, and added to the well of a 96-well plate containing the stimulated macrophages at the effector to target (E:T) ratios indicated in the figures. The cultures were then incubated for an additional 16 hr at 37°, after which the uppermost 50 μl of culture medium was removed from each well, and released radioactivity measured using a Beckman LS5000CE scintillation counter (Beckman Instruments Inc., Fullerton, CA). Cell killing was determined from the ^{51}Cr release calculated using the following formula:

$$\frac{\text{experimental} - \text{spontaneous release}}{\text{maximum} - \text{spontaneous release}} \times 100 = \% \text{ cytolysis},$$

where spontaneous release was evaluated from a sample taken from wells containing target cells only, and maximum release was evaluated from a sample taken from wells containing only target cells to which 20 μl of 10% Triton X-100 had been added 45 min prior to sampling. Whenever nitrite was to be measured, phenol red-free RPMI-1640 supplemented with 5% heat-inactivated FBS (Gibco, Gaithersburg, MD) was used; the presence or absence of phenol red did not appear to influence the extent of cytotoxicity. Spontaneous release was always 25–30% of total counts.

Unless stated otherwise, the results show an average ± standard error of the values obtained from either at least three separate experiments or triplicate samples from one of at least three similar experiments. All reagents were titrated for maximal efficacy and optimal concentrations were used.

RESULTS

Stimulation of NO production by thioglycollate-elicited macrophages from young and old mice with varying concentrations of LPS and IFN-γ

Stimulation of macrophages from young mice with IFN-γ up to 100 U/ml increased their production of NO; 1000 U/ml appeared to be somewhat inhibitory (Fig. 1a). The same was true for stimulation by LPS, where an increase in LPS concentration up to 100 ng/ml increased NO production; 1000 ng/ml appeared somewhat inhibitory except in the absence of IFN-γ. The stimulation pattern for macrophages from old mice was different (Fig. 1b). Stimulation with increasing quantities of IFN-γ resulted in smaller increases in NO production compared with the cells from young mice, and 1000 U/ml caused yet more NO to be produced rather than being inhibitory, as seen with the cells from the young mice. An increase in the concentration of LPS at an IFN-γ concentration of either 0, 10 or 100 U/ml did not appear to have any great effect on NO production. At 1000 U/ml of IFN-γ, an increase in LPS levels did progressively increase NO production up to 100 ng/ml; 1000 ng/ml was inhibitory, however, and even more so than in cells from young mice. From these results, 100 U/ml IFN-γ and 100 ng/ml LPS appeared to be the best combination for macrophages from young mice, while 1000 U/ml of IFN-γ and 100 ng/ml LPS appeared to be the best combination for macrophages from old mice.

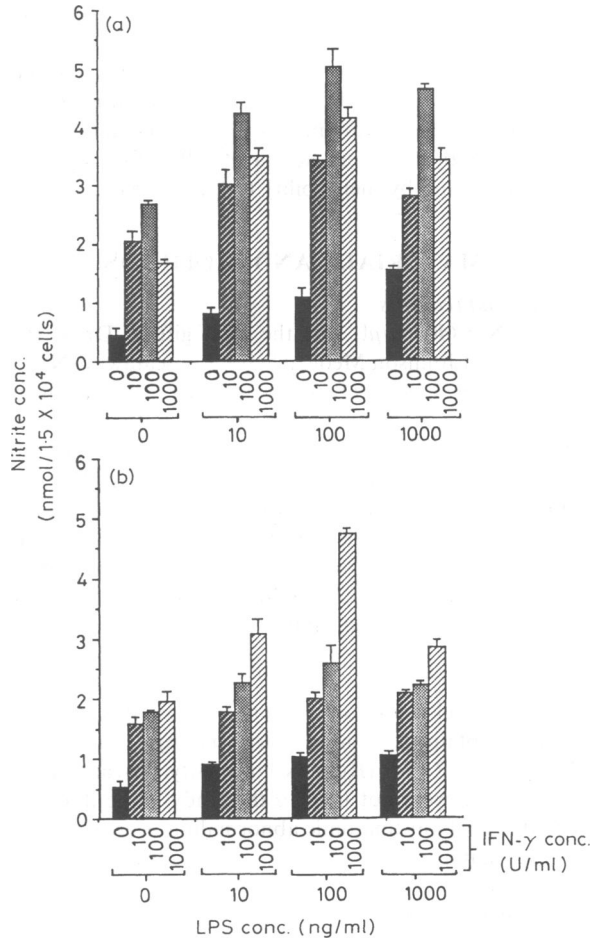

Figure 1. Stimulation of thioglycollate-elicited macrophages with varying concentrations of LPS and IFN-γ. (a) Eight-week-old mice: PEC ($1{\cdot}3 \times 10^5$) from 8-week-old mice were plated into the wells of a 96-well plate. After adherence and washing, approximately 6×10^4 cells (>95% macrophages) were left per well. These cells were then stimulated with varying concentrations of LPS and/or IFN-γ for 24 hr, and a 50-μl sample of each well was analysed for nitrite as described in the Materials and Methods. (b) Two-year-old mice: cells were plated and assayed as for the 8-week-old-mice.

Effect of rOPN on NO production by thioglycollate-elicited macrophages from young and old mice

When stimulated with the optimal concentrations of LPS and IFN-γ for each age group, as determined above, the macrophages from young and old mice appeared to produce about the same amount of NO (Fig. 2a). However, only in the macrophages from young mice was rOPN able to inhibit NO production. To ascertain whether the inhibition of NO production was the result of the suppression of induction of the iNOS mRNA, a Northern blot analysis (Fig. 2b) of the RNA in these cells was performed. As shown in Fig. 2c, the level of iNOS message was twofold higher in activated macrophages from young mice compared with old mice, whereas the OPN message levels were over sevenfold higher in the macrophages from the old mice; these cells also secreted considerably more OPN into the medium than did the young

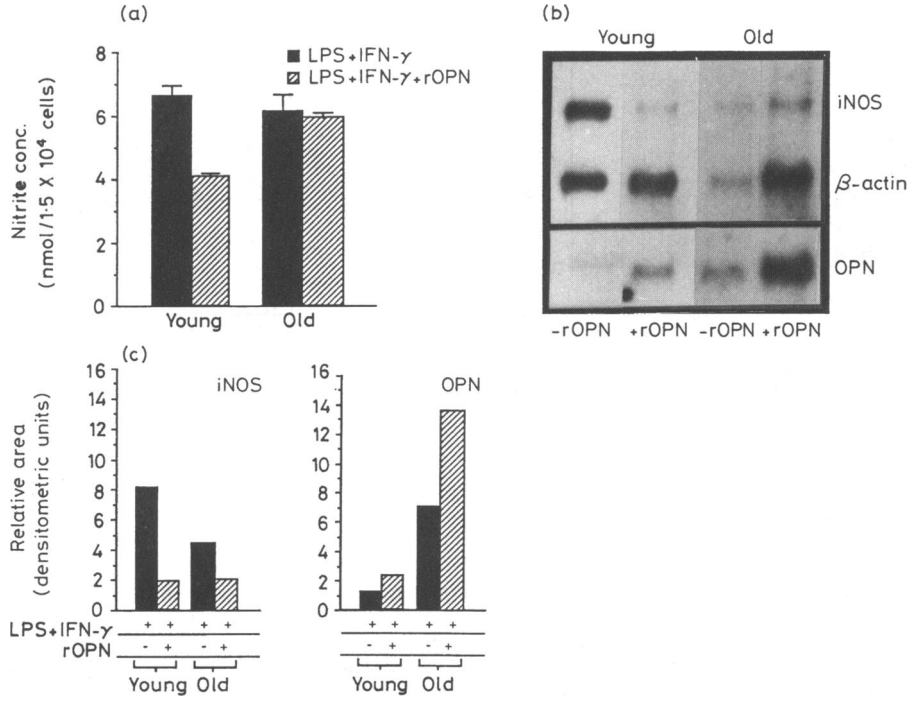

Figure 2. Action of OPN on thioglycollate-elicted macophages from young and old mice. (a) The effect of OPN on NO production by stimulated macrophages. PEC ($1{\cdot}3 \times 10^5$) were plated into the wells of a 96-well plate. After adherence and washing, the remaining adherent cells (> 95% macrophages) were stimulated with either 100 U/ml IFN-γ and 100 ng/ml LPS (macrophages from young mice), or 1000 U/ml IFN-γ and 100 ng/ml LPS (macrophages from old mice). After 24 hr, 50-μl samples of cell-free culture supernatant were analysed for nitrite accumulation. (b) Northern blot analysis of the action of OPN on iNOS mRNA levels. RNA was extracted from parallel sets of cultures similar to those in (a), blotted, and probed for iNOS, β-actin and OPN as described in the Materials and Methods. Although some of the bands in the photograph do not show up well, especially the OPN signal in lane 1, there nevertheless was a clear, though faint, band on the autoradiogram that could be quantified densitometrically. Lane 3 has less RNA than the other lanes because of poor recovery of the RNA. (c) Quantification of the mRNA levels for iNOS and OPN relative to β-actin. These data are derived from a densitometric analysis of the autoradiogram shown in (b).

cells (data not shown). As opposed to the relative ineffectiveness of OPN on NO production by macrophages from old mice, the iNOS message level in the presence of rOPN was decreased by about 50%, suggesting that something other than iNOS mRNA levels was limiting NO production in macrophages from the old mice. The increase in endogenous OPN mRNA levels in the macrophages from the old mice was similar to the increase seen in proximal tubule epithelial cells from the kidneys of older humans compared with cells from kidneys of younger humans.[8]

Effect of age on the cytotoxicity of thioglycollate-elicited macrophages towards tumour cells

When thioglycollate-elicited macrophages from young and old mice were stimulated with 1000 U/ml IFN-γ and 100 ng/ml LPS, the macrophages from old mice appeared less capable of killing the P815 tumour cells than the macrophages from young mice, with the greatest difference in activity between the young and old mouse cells seen at an E:T ratio of 3:1 (Fig. 3). The decrease in cytotoxicity was correlated with a decrease in NO production (Fig. 3). Note that the concentrations of LPS and IFN-γ in this experiment were optimal for NO production by cells from old mice and suboptimal for cells from young mice with regard to IFN-γ concentration. Because the concentration of stimulating agents was optimized for the old mice cells and was somewhat inhibitory to the young mice cells, as determined in Fig. 2a, cytotoxicity assays were also carried out using the macrophages from young mice with the stimulators at their optimal concentrations of 100 U/ml IFN-γ and 100 ng/ml LPS. Under these conditions, cytotoxicity was also closely correlated with NO production (data not shown).

Effect of rOPN on the cytotoxicity of thioglycollate-elicited macrophages from young and old mice towards tumour cells

As illustrated in Fig. 4, there was a dose-dependent inhibition of the cytotoxicity of the effector macrophages from young mice towards the target tumour cells, with the greatest inhibition seen when OPN was added to 0·1 pM. There was a very good correlation between the cell killing and the production of NO (solid squares). The reversal of the effect as the concentration of OPN was raised to higher levels is something that we have observed in a number of contexts (E. E. Rollo manuscript submitted for publication).[28] There are several possible explanations for this kind of dose-dependence, but without additional data we cannot distinguish among them. When the same study was performed with macrophages from old mice, no effect of added OPN was seen at any level on cytotoxicity or NO production.

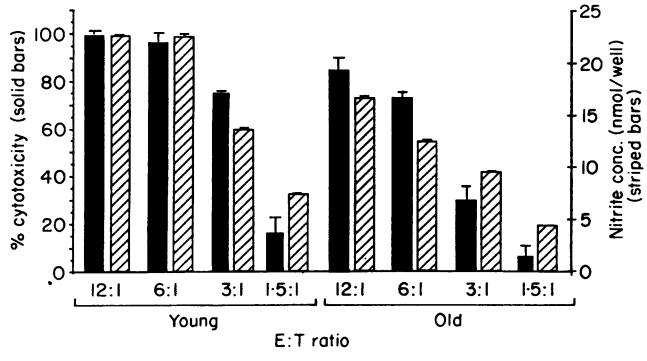

Figure 3. The effect of age on the cytotoxicity of thioglycollate-elicited macrophages towards tumour cells. Left axis: comparison of the cytotoxicity towards tumour cells of macrophages isolated from young and old mice treated with 1000 U/ml IFN-γ plus 100 ng/ml LPS. PEC ($1{\cdot}1 \times 10^5$) from 2·5-year-old and 7-week-old mice were plated into three wells of a 96-well plate, and serially diluted by 2, three times. After adherence and washing, the remaining cells (> 95% macrophages) were stimulated for 8 hr in the presence or absence of 1000 U/ml IFN-γ and 100 ng/ml LPS, and then admixed with ^{51}Cr-labelled P815 mastocytoma cells, at E:T ratios of 12:1, 6:1, 3:1 and 1·5:1. Sixteen hours later, percentage cytotoxicity was determined as described in the Materials and Methods. Cells left unstimulated with medium only showed non-significant NO production or cytotoxicity (data not shown). Right axis: nitrite production by the macrophages from young and old mice. Samples of cell-free medium from the same cultures were analysed for nitrite accumulation. E:T ratio, effector : target all ratio.

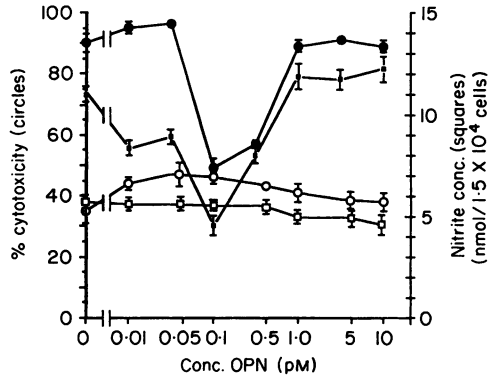

Figure 4. The effect of OPN on the cytotoxicity of and nitrite production by thioglycollate-elicited macrophages from young and old mice. Left axis: the effect of OPN on the cytotoxicity of young (filled circles) and old (open circles) macrophages. PEC ($5{\cdot}4 \times 10^4$) from 8-week-old and 2·1-year-old mice were plated in a 96-well plate, in triplicate. After adherence and washing, the remaining cells were stimulated for 8 hr with 100 ng/nl LPS and 1000 U/ml IFN-γ, in the presence of varying concentrations of recombinant human OPN. ^{51}Cr-labelled P815 mastocytoma target cells were then added at an E:T ratio of 3:1. Sixteen hr later, percentage cytotoxicity was determined, as described in the Materials and Methods. Cells left unstimulated, in the presence or absence of varying concentrations of OPN, showed no significant NO production or cytotoxicity (data not shown). Right axis: the effect of OPN on NO production by macrophages from young (filled squares) and old (open squares) mice. Samples of cell-free medium from the same cultures were analysed for nitrite accumulation.

DISCUSSION

Studies on the metabolic and biochemical differences between young and old animals, or the cells derived from them, reveal a bewildering variety of changes, particularly with regard to the immune system. Although there are some exceptions, older animals and older cells respond less vigorously to any given stimulus than young animals or cells. Our results on the ability of IFN-γ and LPS to stimulate NO production and to activate the cytotoxic activity of macrophages are consistent with this trend—the older cells being less responsive. The most important new conclusion from the research described above is that OPN is less effective at influencing these activities (reducing NO production and inhibiting cytotoxicity) in old cells compared with young cells. We suggest that this is due to the higher levels of OPN found in the older animals and a resulting adaptation that has made the cells less responsive to OPN.

The biphasic dose–response curve (Fig. 4) observed with OPN is not understood. Our working hypothesis is that there is a high-affinity receptor that can respond to low concentrations of added exogenous OPN. The signal generated by this high affinity receptor would be responsible for the suppression of NO production and inhibition of cell cytotoxicity. At higher OPN concentrations we suggest that a second receptor of lower affinity is activated, and that the signal generated by this receptor counteracts the signal generated by the high affinity receptor. Consistent with this hypothesis is the recent report that, in addition to the α_v integrins, CD44 is also a receptor for OPN.[29]

Cytokines that act to diminish and/or terminate the immune response probably serve a protective function by limiting otherwise damaging processes. However, these cytokines may also be exploited by tumour cells and parasites to evade immunity by producing similar mediators or inducing their production by the host, thereby subverting the immune response and making the local environment more permissive. OPN appears to have a role as one of the cytokines that diminishes the immune response through its function as an inhibitor of NO production and of macrophage-mediated tumour cell cytotoxicity. Tumour cells appear to exploit this property of OPN, secreting it at elevated levels in order to inhibit macrophage NO production and the accompanying tumour cell-directed cytotoxicity.[22,30]

Ding *et al.*[5] investigated H_2O_2 and NO production by resident macrophages in response to IFN-γ stimulation, and found the older cells to be less responsive. They also observed that IFN-γ-induced tyrosine phosphorylation of MAPK was reduced in the macrophages from the older mice, even though IFN-γ receptor levels were normal. This is consistent with an impaired signal transduction pathway in the older cells. Recent research has revealed that OPN also stimulates a signal transduction pathway involving protein tyrosine phosphorylation (C. A. Lopez and D. T. Denhardt, manuscript in preparation), and thus the reduced responsiveness of the old cells to OPN may be the result of an impaired signal transduction pathway. Impaired signal transduction could also explain the fact that macrophages from old animals are less cytotoxic towards tumour cells.

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