Free fatty acid release from human breast cancer tissue inhibits cytotoxic T-lymphocyte-mediated killing

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Abstract **Immune-mediated antitumor activities confront a variety of tumor-mediated defense mechanisms. Here, we describe a new mechanism involving FFA that may allow breast cancer to evade immune clearance. We determined the IC_{50} at which unbound free fatty acids (FFA_u) inhibit murine cytotoxic T-lymphocyte (CTL)-mediated killing to assess the physiologic relevance of this phenomenon. We found that the IC_{50} for unbound oleate is 125 ± 30 nM, ~200-fold greater than normal plasma levels. FFA inhibition, however, may play an important role in breast cancer because we found that large quantities of FFAs are released constitutively into the media surrounding samples of human breast cancer but not normal or benign tissue. FFA_u concentration ($[FFA_u]$) increased to at least 25 nM in 20 of 22 cancer tissue samples and exceeded 100 nM in 11 patients. Media from these samples inhibited CTL-mediated killing. Extrapolation from our in vitro conditions suggests that for tumor interstitial fluid, in vivo $[FFA_u]$ may be 300-fold greater than we observed in vitro. Although breast cancer release of FFA may suppress effector cell antitumor activity, strategies that reduce interstitial $[FFA_u]$ may significantly improve antitumor immune therapies.**—Kleinfeld, A. M., and C. Okada. **Free fatty acid release from human breast cancer tissue inhibits cytotoxic T-lymphocyte-mediated killing.** ***J. Lipid Res.*** **2005.** 46: **1983–1990.**

Supplementary key words unbound free fatty acid • free fatty acid release • immunotherapy

Antitumor cytotoxic T-lymphocytes (CTLs) are central to immunotherapeutic anticancer strategies (1–5). Tumor-specific CTLs can be induced by various immunotherapeutic strategies (reviewed in 3), and tumor-infiltrating lymphocytes are found "naturally" in tumors (6). Nevertheless, immunotherapeutic methods are rarely effective in mediating the regression or clearance of established tumors. This lack of effectiveness suggests that factors other than CTL activation may prevent CTL-mediated clearance of tumor in vivo. A number of factors have been identified that reduce CTL effectiveness, including loss of tumor antigen, major histocompatibility complex class I downregulation, and a physical barrier separating CTL and tumor (3). In the present study, we provide evidence for a new mechanism: inhibition of CTL killing of tumor cells by FFAs produced by human breast cancer tumors.

Inhibition of CTL activity by increased levels of total FFAs has been demonstrated in several in vitro studies (7–16). Increased levels of *cis* unsaturated FFAs inhibit CTL signaling pathways and, in particular, inhibit CTL-mediated killing of cognate target cells. These inhibitory effects of *cis* FFAs are attributable to an immediate physical perturbation of the CTL; the effects occur within seconds and are reversed upon extracting FFAs with extracellular albumin. In contrast, saturated FFAs have no effect on signaling or killing. The differential effects of *cis* and saturated FFAs does not involve FFA metabolism because CTL inhibition can be detected before metabolite levels are significant and because the predominant metabolites of oleic acid (the most potent FFA inhibitor) cannot be extracted with extracellular albumin (9). The differential effects of *cis* and saturated FFAs, however, are well correlated with differential effects of *cis* and saturated FFAs on membrane lipid order (10, 14), consistent with a physical mechanism of FFA-mediated CTL inhibition.

In biological fluids, most FFA is bound to albumin and/or cells (17, 18). Although only a small fraction of total FFA is unbound free fatty acids (FFA_u) (19), it is the FFA_u that determine the degree of FFA inhibition of cellular function (14). FFA_u levels that inhibit CTL-mediated killing have not been determined. Estimates have been obtained for FFA_u levels (IC_{50}) that inhibit signaling in murine CTL, in this case the concanavalin A-stimulated increase in intracellular calcium (14). The IC_{50} for signaling inhibition is FFA type-dependent and is ~200 nM for oleate, probably the most important FFA for suppression of the CTL response in vivo because it is the most abun-

Abbreviations: ADIFAB, acrylodan-labeled intestinal fatty acid binding protein; CHTN, Cooperative Human Tissue Network; CTL, cytotoxic T-lymphocyte; FAFBSA, fatty acid-free bovine serum albumin; FCS, fetal calf serum; FFA_u, unbound free fatty acids; $[FFA_u]$, unbound free fatty acid concentration; OA_u, unbound oleate concentration.

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dant *cis* unsaturated FFA. Normal human plasma total FFA_u levels are ~1.4 nM (20), of which ~0.5 nM is oleate; therefore, normal unbound oleate levels are 400-fold smaller than the in vitro IC_{50} for oleate inhibition of signaling.

Specific physiologic conditions, however, may increase plasma FFA_u levels sufficiently to inhibit CTL activity. Lipid/heparin infusions in healthy volunteers generate plasma FFA increases that inhibit T-cell signaling (16), and plasma FFA_u levels can exceed the IC_{50} for CTL signaling in acute cardiac ischemia (20, 21). Increased plasma FFAs also occur in the cachexia that is often associated with cancer (22), and lymphocyte function is inhibited in cancer patients with increased plasma FFA levels (23). Because FFA_u increases exponentially with increasing total FFA to albumin (17, 18), total FFA increases of 4- to 6-fold above normal, as observed in the studies described above, would be expected to generate inhibitory levels of FFA_u.

The mechanisms described above for increasing plasma FFA reflect the mobilization of FFA from adipose storage. FFAs may also be generated by activation of lipolysis in the tumor cells themselves. In vitro studies reveal that high levels of FFA are released from tumor cells within minutes of CTL attack (24, 25). In the present study, we monitored the constitutive FFA release from human breast tissue samples and determined the effect of this release on murine CTL killing of cognate target cells. Our results, which are the first to demonstrate that FFAs are released from breast cancer tissue, also show that no release occurs from benign tumor and normal breast tissue. In addition, we determined the dose response for FFA_u inhibition (IC_{50}) of CTL-mediated killing of cognate tumor cells and found that FFA_u levels produced by breast cancer tissue can exceed the IC_{50}. Our results suggest that constitutive FFA release in vivo may allow breast cancer tumors to evade clearance by CTL.

METHODS

CTL clones and tumor cell lines

Maintenance and stimulation of murine CTL clones and tumor cell lines was performed as described previously (26). The CTL clone C30 (anti-H2-K^b) was a gift from Dr. Linda Sherman (Scripps Institute of Research, La Jolla, CA), and the CTL clone OE4 (anti-$H2^d$) was a gift from Osami Kanagawa (Lilly Research Laboratories, La Jolla, CA). The CTLs were maintained in vitro by weekly stimulation with irradiated C57BL/6J and BALB/CJ spleen cells, respectively, and propagated in RPMI 1640 plus 10 mM HEPES and 4 mM L-glutamine (all from Cambrex Bio Sciences), 1% MEM nonessential amino acid solution and 1 mM sodium pyruvate (both from Sigma), 10 U/ml recombinant interleukin 2 (National Institutes of Health), 50 mM 2-mercaptoethanol (Serva Feinbiochemica), and 10% heat-inactivated fetal calf serum (FCS; Tissue Culture Biologicals, Tulare, CA). The respective allogeneic cognate tumor target cells, EL4 (H-$2K^b$) and P815 (H-$2K^d$), were maintained in vitro in the same media as for CTL with 2.5% FCS and 7.5% calf serum instead of 10% FCS and were periodically restarted from frozen stock.

Assay for lytic activity

Lytic activity was measured by the release of ^{51}Cr from target cells loaded with $Na_2{}^{51}CrO_4$ (Perkin-Elmer Life Sciences Products) as described previously (11). CTL-target cell conjugates, at a total volume of ~200 μl on 96-well plates, were formed by centrifugation using a 5:1 ratio of CTL to target cells (5×10^3 cells) in a buffer composed of 20 mM HEPES, 140 mM NaCl, 5.5 mM glucose, 5 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgSO_4$ at pH 7.4 (C-HEPES) for 5–10 min at 1,000 rpm (160 *g*). Harvest of the supernatant was done after 4 h of incubation at 37°C, which was then assayed for ^{51}Cr release. Parallel samples containing labeled target cells with buffer in place of CTL and appropriate amounts of testing sample were assayed in the same way to yield a value for spontaneous release. Total counts were determined from ^{51}Cr released upon treatment of the labeled target cells with 2% Triton X-100. Specific lysis was calculated as 100 × (sample spontaneous release)/(total spontaneous release). Measurements for each assay condition were performed using between three and five replicates. The reported values are averages and standard deviations of these replicates.

Breast tissue preparation

Breast tissue samples obtained immediately after surgery from the Cooperative Human Tissue Network (CHTN) were shipped on ice overnight in RPMI 1640 containing penicillin and streptomycin. The protocol for the use of these samples was approved by the Torrey Pines Institute for Molecular Studies Institutional Review Board, and patient informed consent was obtained by the participating CHTN hospitals. Histological analysis was performed by pathologists at the CHTN participating hospitals. Breast tissue samples included 22 cancer samples and, for 14 of these, a matched normal and/or benign tissue sample obtained from the margins of the excised tumor where no carcinoma was detected. Pathology reports indicated that >70% of the cancer samples were ductal carcinomas; the remaining samples were lobular or untyped mammary carcinomas, and metastases were found in ~60% of patients.

Tissue sample weights ranged from 0.3 to ~3 g. Samples were cut into pieces of ~0.25 g after visible adipose, connective, and necrotic tissue had been removed. For the 14 matched samples, the weight (after removal of adipose, connective, and necrotic tissue) of the cancer tissue was less than or equal to that of the matched normal/benign tissue. Each piece of tissue was incubated at 37°C in a CO_2/H_2O-controlled atmosphere in 15 ml of media containing MEGM (Clonetics Mammary Epithelial Cell Medium with growth factors, cytokines, and supplements; Cambrex, Walkersville, MD) with 10% FCS (Tissue Culture Biologicals) plus penicillin, streptomycin, and gentamycin. Approximately every 1–2 days, for up to 7 weeks, 180–200 μl of the incubation medium was collected, centrifuged to remove cellular debris, and stored at −80°C. The medium remaining at the end of the incubation period was also collected and stored similarly.

FFA_u treatment

Cells were treated with defined concentrations of FFA_u using FFA-BSA complexes to buffer FFA_u as described previously (27). FFA-BSA complexes were formed by titrating solutions of 600 μM BSA (fatty acid-free from Sigma) in C-HEPES with sodium salts of the FFA (Nu-Chek Prep, Elysian, MN). The FFA_u concentration ($[FFA_u]$) for each complex was determined using the fluorescent probes acrylodan-labeled intestinal fatty acid binding protein (ADIFAB) or the L72A mutant (ADIFAB2) (FFA Sciences LLC) as described previously (28, 29).

FFA treatment of CTL was performed by suspending the CTL in C-HEPES, generally at 2.5×10^5 cells/ml, to which the appropriate (defined $[FFA_u]$ value) FFA-BSA complex was added to make

the final BSA concentration, in the CTL-target mixture, between 60 and 300 μM. The CTLs were incubated in this medium for ~10 min at 37°C before the formation of CTL-target cell complexes. After the 4 h killing assay, FFA_u levels were measured in the CTL-target and the target (spontaneous release) media. The ADIFAB fluorescence measurements used ~150 μl of media to 1.35 ml of C-HEPES, yielding a final [BSA] of ⩾6 μM. Under these conditions the FFA_u provides an accurate estimate of the undiluted media (20).

With sufficiently high [BSA], the FFA_u levels generated by the FFA-BSA complexes should be well buffered (invariant) with or without cells present (30). In the present studies, we found that the degree of [FFA_u] buffering on the plastic 96-well plates used for the cytolysis assays was FFA type-dependent. For oleate and linoleate, the difference in [FFA_u] values with or without (FFA-BSA complexes only) cells present was not significant. Moreover, for oleate and linoleate, [FFA_u] values for FA-BSA complexes was virtually identical on 96-well plates and in cuvettes. However, for palmitate, the combined effects of binding to the well surfaces and cells was significant and limited the maximum achievable FFA_u level to ~200 nM.

CTL-target cell complexes were also treated with media from normal, benign, and cancer breast tissue obtained at the end of the incubation period. This medium, which contains 10% FBS, is ~60 μM albumin. To maximize the FFA-BSA buffering capacity, the volume of breast tissue medium added to the CTL-target cell complexes in C-HEPES was between 60% and 90% of the total volume, yielding ~40–50 μM albumin. The actual [FFA_u] values of the breast tissue/CTL-target cell complexes were determined at the completion of the killing assay by direct measurement of media samples, as described previously for plasma (20). These [FFA_u] values are the sum of concentrations of the five different FFAs shown in Fig. 4 below that are released into the media. We estimate, using albumin binding constants, that the fraction of *cis* [FFA_u] is ~75% of the total [FFA_u], greater than the 60% of total FFA (see Fig. 4) because of the higher affinities of the saturated FFAs.

Fatty acid analysis of breast tissue media

Sample aliquots of 1.0–9.0 ml of breast tissue media were extracted for lipid analysis using chloroform-methanol as described previously (25). The extract was dried under nitrogen, resuspended in chloroform, and fractionated by silica gel TLC using petroleum ether-ethyl ether-acetic acid (80:20:1) as the developing solvent. TLC plates were stained with iodine, and the FFA fraction was eluted, dried under nitrogen, and resuspended in 50–100 μl of ethanol.

The extracted FFAs were analyzed using an HP-5890 gas chromatograph equipped with a 10 m HP-FFAP capillary column (Agilent Technologies, Wilmington, DE). Detector and injector temperatures were 250°C, and helium was the carrier gas. FFAs were eluted from the column using a splitless injection technique and a three-step temperature ramp from 180°C to 240°C. Chromatograms were referenced to standards (Nu-Chek Prep) and analyzed using Agilent ChemStation software.

RESULTS

CTL-mediated killing is inhibited at FFA_u levels that greatly exceed normal plasma concentrations

Previous studies have demonstrated that CTL-mediated killing of target cells was inhibited at high levels of total FFAs (7, 8, 11, 16). To assess the physiologic relevance of FFA-mediated inhibition, it is necessary to determine the

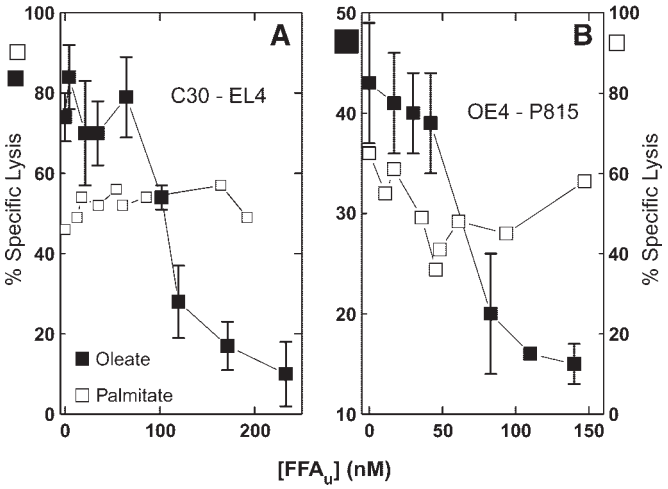

Fig. 1. Dose dependence for unbound free fatty acid (FFA_u) inhibition of cytotoxic T-lymphocyte (CTL)-mediated killing. Specific lysis of ^{51}Cr-labeled murine tumor targets EL4 and P815 by their cognate CTLs, C30 and OE4, respectively, was measured as a function of the unbound concentrations of oleate and palmitate. FFA_u concentration ([FFA_u]) levels in the CTL-target cell suspensions were maintained by FFA-BSA complexes for which [BSA] was >60 μM. A: C30 CTL killing of EL4 cells. The specific lysis scales for oleate and palmitate are the same. B: OE4 killing of P815 cells. The specific lysis scale for oleate is indicated on the left and that for palmitate on the right vertical axis. The average specific lysis (percent) and standard deviations from three to five replicates for each [FFA_u] (in nM) are shown for oleate; for ease in presentation, only the average values are shown for palmitate. The standard deviations for palmitate are similar to those for oleate and average 14%.

[FFA_u] dose dependence, which was not possible in these earlier studies. We have reexamined the inhibition of CTL-mediated killing by FFA and used the ADIFAB method to determine the FFA_u dose dependence for inhibition.

We determined the effect of unbound oleate, linoleate, and palmitate on the degree of target cell lysis mediated by two different murine cognate CTL-target systems: C30 (anti k^b)-EL4 (k^b) and OE4 (anti k^d)-P815 (k^d). CTLs were incubated for 5–10 min before target cell conjugation with FFA-BSA complexes designed to maintain the extracellular media at defined [FFA_u]. Measurements of CTL-mediated lysis were performed by assessing ^{51}Cr release from target cells with increasing [FFA_u] in the cell media (see Methods).

Our measurements of ^{51}Cr release reveal that CTL-mediated lysis decreases with increasing unbound oleate concentration ([OA_u]) (**Fig. 1**). The average IC_{50} for inhibition was ~120 nM for both CTL-target cognate pairs (**Table 1**). Results for linoleate revealed a similar behavior but with a larger IC_{50} of ~250 nM (Table 1). Palmitate, in contrast to the *cis* unsaturated oleate and linoleate, caused no inhibition up to the maximum unbound palmitate level achievable (Fig. 1). Inhibition by *cis* unsaturated FFA_u but not palmitate is consistent with previous results for total FFAs (11, 16). However, the concentrations of *cis* FFA_u that generate significant inhibition are much greater than the molecular species average [FFA_u] of 1.4 nM in normal human plasma (95th percentile ~ 2.5 nM) (20). Thus, inhi-

TABLE 1. IC_{50} values for oleate and linoleate inhibition of CTL-mediated killing

	Fatty Acid			
	Oleate		Linoleate	
CTL	IC_{50}	n	IC_{50}	n
C30	121 ± 26	13	230 ± 50	4
OE4	127 ± 24	6	230 ± 50	4

CTL, cytotoxic T-lymphocyte. Values for IC_{50} are averages and standard deviations of n experiments.

bition should not occur in plasma under normal physiologic conditions.

For each [FFA_u], the level of ^{51}Cr release was also measured from a matched quantity of target cells without CTL. This "spontaneous" release was subtracted from the total ^{51}Cr release to obtain the level of specific lysis. We observed an increase in spontaneous lysis at [OA_u] or linoleate concentration significantly greater than the IC_{50} values (data not shown). In contrast to target cells, CTL revealed little or no lysis (as monitored by trypan blue) at these higher [FFA_u] values.

Inhibition can be reversed by removing FFA_u

Our previous studies indicated that FFA-mediated inhibition of CTL signaling could be reversed by reducing FFA_u levels (9). Therefore, we investigated whether inhibition of CTL-mediated lysis can also be reversed by reducing [FFA_u]. To do this, we carried out [FFA_u] titration experiments similar to those used in Fig. 1, but for each [FFA_u] we added fatty acid-free bovine serum albumin (FAFBSA) to the CTL-target sample at increasing times after CTL-target conjugate formation. The results indicate that inhibition is reversed, and lysis is restored, if FAFBSA is added within 1.5 h of conjugation (**Fig. 2** shows the results for FAFBSA addition at 45 min). The apparent impairment of reversibility at longer times may simply reflect the resetting of the "clock" for lysis after FAFBSA addition, thereby effectively reducing the assay period. Adding FAFBSA within 2 h from the start of the incubation also blocked the increase in spontaneous target cell lysis at higher [FFA_u] (data not shown). This indicates that CTLs remain functional if FFA_u is removed and that even relatively high FFA_u levels do not significantly lyse targets or CTLs.

Large quantities of FFAs are constitutively released from breast cancer but not normal breast tissue

Because we observed previously that CTL attack activates FFA release from cognate tumor cells (25), we speculated that rapidly metabolizing tumor cells might release significant levels of FFAs. In this event, FFA_u levels might be increased sufficiently in the interstitial fluid surrounding the tumor to interfere with CTL clearance of the tumor. We investigated whether FFA was released from human breast tissue by monitoring the time course of FFA_u levels in the media surrounding the tissue (see Methods). This approach for investigating breast tumor interstitial fluid is similar to that described recently for the proteomic characterization of this fluid (31).

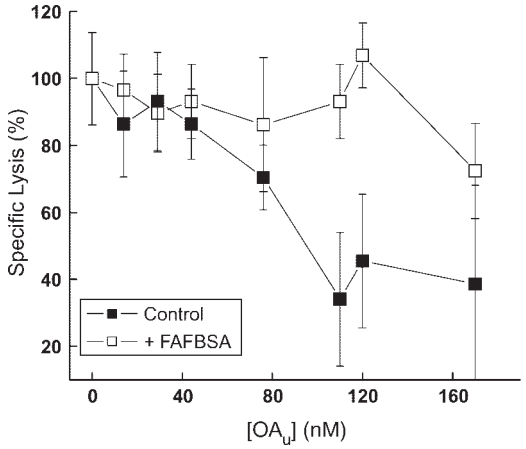

Fig. 2. Reversibility of FFA_u inhibition. Specific lysis of EL4 cells by C30 CTLs as a function of unbound oleate concentration ([OA_u]) either with (open squares) or without (closed squares) the addition of 120 μM fatty acid-free bovine serum albumin (FAFBSA) at 45 min after C30-EL4 conjugation. Values were normalized to specific lysis at zero [OA_u], which was 30% and 45% for cells to which FAFBSA was or was not added, respectively. [OA_u] was determined in cells without added FAFBSA. Values shown are averages ± SD.

We found that [FFA_u] increased significantly (ΔFFA_u > 10 nM) for 19 of the 22 cancer samples but in only 2 of the normal samples and in neither of the 2 benign tumor samples (**Fig. 3** and data not shown). The difference in tumor/normal breast tissue FFA release is illustrated in Fig. 3 for six of the matched tumor/normal tissue pairs. Although the magnitude and rate of FFA release varied considerably for different patients, most cancer samples revealed significant (ΔFFA_u > 10 nM) FFA_u increases within the first 2 days and most increased to 50 nM or greater within ~10 days. As explained in Discussion, these in vitro levels likely represent a severe underestimate of actual tumor interstitial fluid levels. Normal FFA_u levels did not change with time and were generally <5 nM. These results suggest that FFA_u released by the cancer tissue is an intrinsic property of the malignant cells, because little or no FFA was released from normal tissue, although the mass of normal tissue was greater than or equal to that of the cancer tissue (see Methods).

Evidence supporting the reliability of the ADIFAB determination of [FFA_u] was obtained by repeating the [FFA_u] measurements after adding 10 μM FAFBSA to each sample. In every case, [FFA_u] decreased significantly, especially for samples with high pre-FAFBSA FFA_u levels (see Fig. 5 below). This is consistent with the expected nonlinear dependence of [FFA_u] on the total FFA-BSA ratio at high [FFA_u] (17, 18).

We used gas chromatography to determine the distribution of molecular species of FFAs released from several cancer and normal tissues after lipid extraction and thin-layer chromatography of the respective media. The results indicate that the distribution of the major FFAs was approximately similar in cancer and normal tissue (**Fig. 4**). The predominant FFA released from breast cancer tissues is oleic acid, constituting ~30–40% of the total. As expected

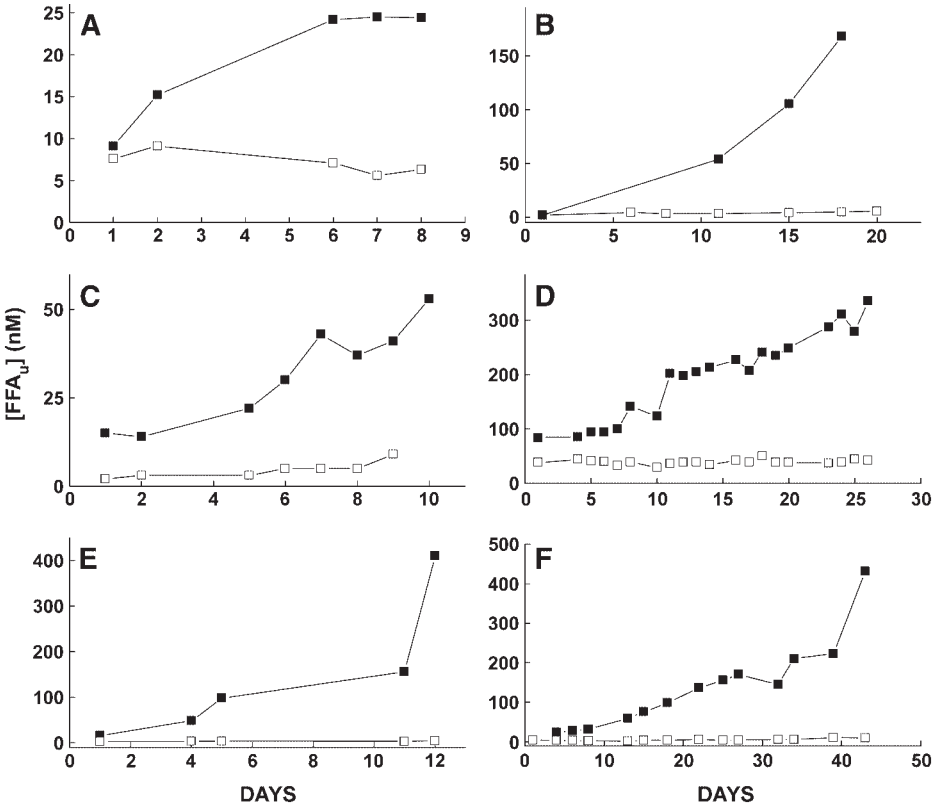

Fig. 3. Time courses for FFA release from cancer and matched normal breast tissues. [FFA_u] was measured in the tissue media for matched cancer (closed squares) and normal (open squares) breast tissue samples from six different patients (A–F). Results shown are averages of two or more measurements, and the standard deviations were ~5%.

from the FFA_u results, the mass of FFA in cancer media was considerably (>10-fold) greater than in normal media.

Media from breast cancer but not normal tissue inhibits CTL-mediated lysis

Because increased levels of *cis* FFA_u inhibit CTL-mediated killing and *cis* FFA_u levels are increased in breast cancer tissue media, we compared CTL-mediated killing of EL4 and P815 cells in media from normal, benign, and cancer breast tissue. The media used were obtained from the last day of incubation, generally when [FFA_u] in the cancer medium had achieved its maximum value. The results of these measurements indicate that media from breast cancer tissue, with high [FFA_u], but not from normal or benign breast tissue inhibit CTL-mediated lysis, as indicated in **Fig. 5** for C30 killing of EL4 cells. Inhibition was greater in the cancer media compared with OA-BSA because of the large fraction of *cis* FFA_u in the media (see Methods). Figure 5 also shows that inhibition can be reversed by reducing FFA_u levels with FAFBSA. These results support FFAs as the mediators of inhibition by breast cancer media.

DISCUSSION

In this study, we provide evidence that FFA release from human breast cancer tumors represents a previously unrecognized mechanism by which tumors may evade immune-mediated clearance. This conclusion derives from our observation that large quantities of FFAs were released constitutively from human breast cancer tissue but not from benign tumor or normal tissue. FFA_u levels in cancer tissue media often exceeded 120 nM, the IC_{50} for oleate inhibition of murine CTL-mediated killing of cognate tumor cells, and oleate was the most abundant FFA_u released from the breast cancer tissue. Media from cancer but not normal or benign tissues inhibited CTL-mediated killing of cognate tumor cells, and this inhibition could be reversed by reducing FFA_u levels. These observations suggest that [FFA_u] is increased in the interstitial fluid of breast cancer tumors and that reducing [FFA_u] might improve the efficacy of immune-mediated clearance of the tumors.

The effects of FFAs released from tumor cells have not been reported previously. Earlier studies, however, have described relationships between exogenously added FFAs and tumor proliferation. For example, exogenously added arachidonate was found to inhibit the growth of breast cancer and colorectal cancer cell lines in vitro, whereas linoleate uptake stimulated the growth of a rat hepatoma in vivo (32–35). A complication in interpreting results derived from exogenously added FFAs, as emphasized by Hardy, Langelier, and Prentki (35) and the present study, is that without careful control of [FFA_u], the physiologic

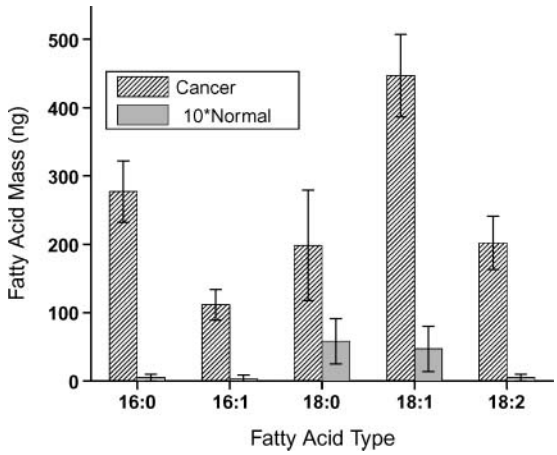

Fig. 4. Distribution of FFAs released from cancer and normal tissues. Gas chromatography was used to determine FFA distribution in cancer and matched normal breast tissue media. The FFA fraction was obtained from tissue media taken from the last day of the incubation period (generally when [FFA_u] was greatest). Results are averages and standard deviations of three separate determinations for each sample, and the normal sample results are 10 times the measured values. [FFA_u] values were 4 and 410 nM for the normal and cancer samples, respectively. FFAs are designated by number of carbons and double bond number. The corresponding trivial names are as follows: palmitate (16:0), palmitoleate (16:1), stearate (18:0), oleate (18:1), and linoleate (18:2).

relevance of such studies cannot be gauged accurately. In the study of Hardy, Langelier, and Prentki (35), for which FFA/albumin ratios were maintained at physiologic levels, oleate stimulated proliferation but palmitate inhibited growth of the MDA-MB-231 breast cancer cell line in vitro. In addition, Hardy, Langelier, and Prentki (35) and others

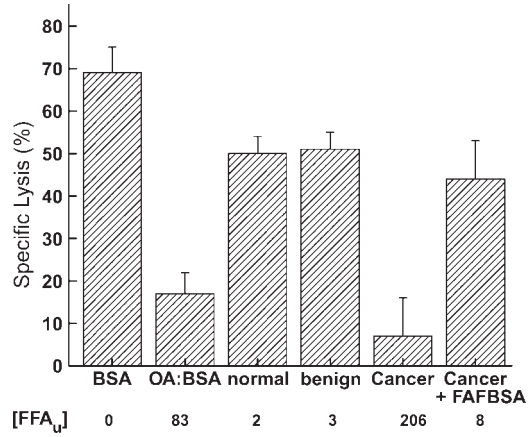

Fig. 5. Effect of breast tissue media on CTL killing. C30-CTL killing of EL4 cells was measured in the following media with corresponding [FFA_u] (total of all FFA_u) and percent lysis: first bar, C-HEPES (60 μM FAFBSA, [FFA_u] = 0, 69 ± 6%); second bar, C-HEPES plus oleate/BSA (60 μM BSA, [OA_u] = 83 nM, 17 ± 6%); third bar, normal breast tissue media ([BSA] ≈ 45 μM, [FFA_u] = 2 nM, 50 ± 4%); fourth bar, benign breast tissue media ([BSA] ≈ 45 μM, [FFA_u] = 3 nM, 51 ± 4%); fifth bar, cancer breast tissue media ([BSA] ≈ 45 μM, [FFA_u] = 206 nM, 7 ± 9%); and sixth bar, cancer breast tissue media plus 60 μM FAFBSA ([BSA] ≈ 105 μM, [FFA_u] = 8 nM, 44 ± 9%).

(36) have found that oleate protects cells against palmitate-mediated apoptosis. This may be relevant to the results of the present study, because both in CTL-mediated tumor cell release of FFA (25) and in the constitutive release from breast cancer tissue (Fig. 4), significantly more oleate than palmitate is released.

Additional evidence that FFAs are essential to tumor growth derives from studies demonstrating that FAS is highly expressed in many tumor cells, including breast cancer, and that inhibition of FAS inhibits tumor growth (37). These studies are consistent with the notion that high levels of FFA synthesis are necessary for the viability of rapidly metabolizing cancer cells. This high level of metabolism might also be needed to replenish the large quantities of FFAs that we find secreted from tumor cells. However, we did not find, in studies of selected tissue samples, that the FAS inhibitor C57 reduced the level of FFA_u secreted from breast cancer tissue (data not shown).

Except for adipocytes, fatty acid release from nontransformed cells appears to occur only upon loading cells with triacylglycerol, and even then, net release requires virtually zero extracellular FFA_u (38). Ehrlich ascites tumor cells release FFAs without loading, but net release also occurs only when extracellular FFA_u is virtually zero (39). In contrast, net release from breast cancer, but not benign or normal tissue, increases virtually independently of extracellular FFA_u levels (Fig. 3).

The difference in FFA release from cancer and normal/benign tissue is likely a reflection of tumorigenesis rather than, for example, a different distribution of normal cells in the different tissues. Breast tissue is predominantly a mixture of adipose, fibroblastic, epithelial, and myoepithelial cells. Our results were based on samples from 22 different patients (22 cancer and 14 matched normal/benign), each of which is a mixture of the different cell types. All cancers were of epithelial origin (carcinomas), which are also constituents of the normal/benign samples. Thus, FFA release from cancer tissue is likely a reflection of epithelial cell transformation.

Most of the FFAs released from breast cancer tissue probably originates from cellular membranes. Microscopic examination of dispersions of cells from selected breast tumor samples did not reveal significant amounts of intracellular lipid droplets (data not shown). Previous studies indicate that the phospholipid content is 4-fold higher and the triacylglycerol content is 65% lower in cancer compared with noncancerous parts of the breast tissue (40). Moreover, the fatty acid composition of the cancer tissue phospholipids was highly enriched in unsaturated FFAs compared with noncancerous tissue, whereas triacylglycerol revealed no significant difference between cancer and normal tissue (40). Consistent with these observations, *cis* unsaturated FFAs are the predominant FFAs released from breast cancer tissue (Fig. 4). Our earlier study of CTL-mediated FFA release from murine tumor cell lines also revealed a phospholipid origin of the released FFAs and enrichment of *cis* unsaturated FFAs (25). Thus, our earlier study and the present study are consistent with a FFA release mechanism involving phospholipase activity.

Although phospholipid may be the source of the FFAs, the mechanism that activates lipolysis and the mechanism that releases FFAs from the tumor tissue are unknown. Necrotic tissue is present especially in high-grade breast cancer lesions and is thought to be induced by hypoxia within the tissue (41). Loss of membrane integrity in necrotic cells would increase intracellular calcium levels and thereby activate calcium-sensitive phospholipases. FFAs might then be released from the cells simply by diffusing through the membrane defects created during necrosis. Alternatively, FFAs may be released from the cell by transport through an intact membrane. To achieve high extracellular [FFA_u] would require high (>50 nM) intracellular [FFA_u] and/or an ATP-driven FFA pump having an orientation opposite the one we have described in adipocytes (30, 42). Determination of the specific mechanism is important for developing methods to reduce release, in the event that this phenomenon can be confirmed in vivo. Future studies will attempt to determine the mechanism of breast tumor release of FFAs.

The results of this study suggest that FFA_u levels in the interstitial fluid surrounding a breast cancer tumor may be increased sufficiently to inhibit the cytolytic activity of infiltrating CTLs. To estimate in vivo interstitial FFA_u levels from our results, we compared the FFA-buffering capacity of the in vitro extracellular media with that expected in interstitial fluid in vivo. In our measurements, the volume of extracellular media was ~60-fold greater than the volume of tissue mass (15 ml to 0.25 g; see Methods). Measurements of the ratio of interstitial volume to adipose and skeletal muscle tissue volume yield ~0.1 (43), ~600-fold less than in our in vitro measurements. On the other hand, the interstitial albumin concentration (44), ~130 μM, is roughly twice the 60 μM (10% FCS) seen in our extracellular media. Combining these two factors suggests that the buffering capacity of interstitial fluid is ~300-fold less than in our extracellular media. Therefore, if in vivo tumor production and efflux of FFAs are similar to those of isolated tissue, the magnitude and rate of increase of FFA_u levels may be significantly (up to 300-fold) greater in interstitial fluid than we observe in vitro.

It follows that at relatively early stages in tumor development, interstitial FFA_u levels may be increased sufficiently to inhibit CTL activity. Even if activated CTLs are generated and are able to infiltrate a tumor, increased interstitial FFA_u levels may prevent CTL-mediated clearance of the tumor. Because CTLs also stimulate very rapid and large amounts of FFA release from cognate tumor targets (24, 25), any level of CTL-mediated killing may actually further increase interstitial FFA_u levels. Plasma FFA_u may also be increased in cachexia, diabetes, and cardiac ischemia (FFA release from the tumor, because of its small mass, probably does not contribute significantly to plasma [FFA_u]). Any increase in plasma FFA_u would further diminish the buffering capacity of the interstitial fluid and thereby enhance immune suppression. If in situ tumor interstitial fluid [FFA_u] is increased and if therapeutic strategies can be developed to limit the tumor release of FFAs, the success of immune-mediated tumor clearance may be improved.

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