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27. This behavior is consistent with our observation that the addition of polar organic solvents such as acetone or ethanol to the latex emulsion induces latex coagulation, but addition of water alone does not produce this effect (*26*).
28. We thank Mexico's Consejo de Arqueologia at the Instituto Nacional de Antropologia e Historia (INAH) and J. Garcia-Bárcena, C. Rodriguez, and P. Ortiz of INAH for permissions to perform this study; in Chiapas, J. Gasco, F. Guillen, L. Guillen, and A. Castañeda; in the United States, C. Coggins; at Harvard University, the staff at the Peabody Museum and Botany libraries; and V. Williams, M. Rubner, C. Scott, W. Williams, H. Lechtman, and the Undergraduate Research Opportunities Program at the Massachusetts Institute of Technology.

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Requirement for Croquemort in Phagocytosis of Apoptotic Cells in *Drosophila*

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Macrophages in the *Drosophila* embryo are responsible for the phagocytosis of apoptotic cells and are competent to engulf bacteria. Croquemort (CRQ) is a CD36-related receptor expressed exclusively on these macrophages. Genetic evidence showed that *crq* was essential for efficient phagocytosis of apoptotic corpses but was not required for the engulfment of bacteria. The expression of CRQ was regulated by the amount of apoptosis. These data define distinct pathways for the phagocytosis of corpses and bacteria in *Drosophila*.

Phagocytosis is the terminal event of the apoptotic process (*1*, *2*) and is also critical for the engulfment of microorganisms (*3*). It has been proposed that the recognition of both nonself (microorganisms) and effete self (corpses) may share common receptors (*4*). Blocking experiments have implicated a number of receptors as important for target recognition (*2*–*4*). Genetic studies indicate that some of these receptors participate in phagocytosis of pathogens in vivo (*5*, *6*). However, the multiplicity and redundancy of recognition mechanisms in mammalian systems have made it difficult to evaluate the relative roles of these receptors in the phagocytosis of corpses. Although several genes of *Caenorhabditis elegans* are involved in the phagocytosis of corpses (*7*–*9*), none of these molecules seem to act directly as a receptor in the recognition of the corpse.

In *Drosophila* embryos, like in mammals and in contrast to worms, the clearance of apoptotic cells is primarily mediated by macrophages, hemocytes that become phagocytic at the initiation of developmentally regulated apoptosis (*10*). Croquemort (CRQ), a *Drosophila* CD36-related receptor, is specifically expressed on all embryonic macrophages (*11*). Human CD36 acts as a scavenger receptor (*12*–*14*) and also binds apoptotic cells in combination with the macrophage vitronectin receptor and thrombospondin (*15*, *16*). CD36 has the ability to confer phagocytic activity on nonphagocytic cells on transfection (*17*, *18*). CRQ expression in nonphagocytic Cos7 cells allows these cells to recognize and engulf apoptotic thymocytes (*11*). Thus, CRQ may participate in the removal of apoptotic cells during *Drosophila* embryogenesis. We genetically evaluated the relative

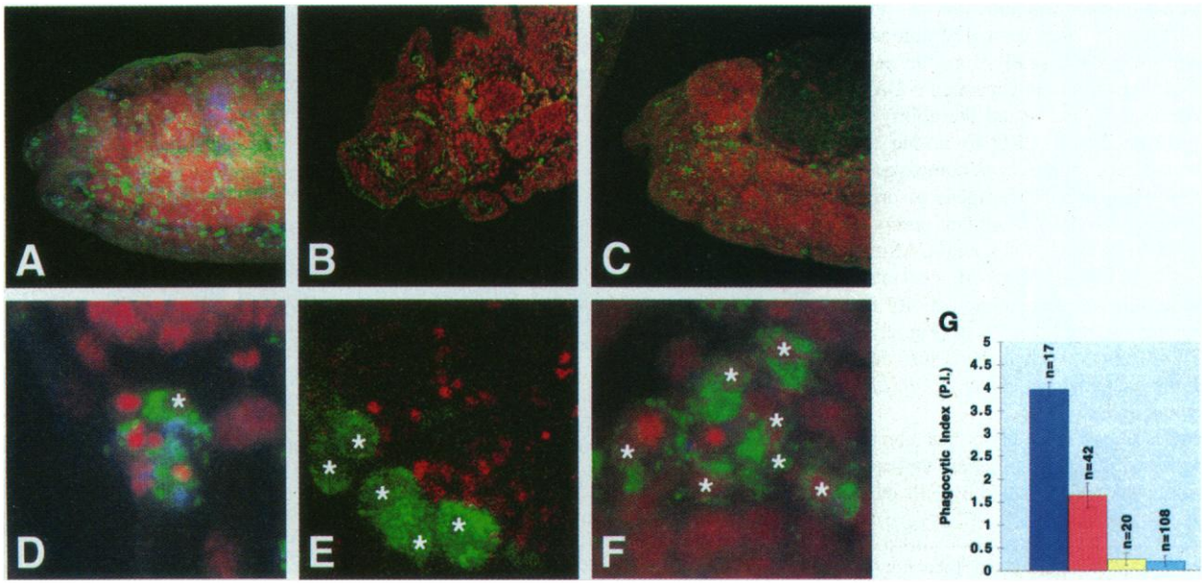

Fig. 1. Macrophages in *crq*-deficient embryos have very poor phagocytic activity for apoptotic cells. (**A** to **F**) In confocal micrographs, peroxidasin-stained hemocytes appear green, CRQ staining appears blue, 7-AAD–stained apoptotic corpses appear as bright red round particles, and the nuclei of viable cells appear as large red diffused components. All images are the sum of eight focal planes. (A) to (C) show a ×40 magnified lateral view of the head region of (A) a *In(2L)Cy* homozygous embryo, (B) a *Df(2L)al* homozygous embryo, and (C) a *W88* homozygous embryo. (D) to (F) show high-magnification views (×400) of their respective macrophages. As compared with the wild-type distribution (A) and phagocytic activity (D) of macrophages within *In(2L)Cy* homozygous embryos, macrophages in *Df(2L)al* (B) and *W88* (C) homozygous embryos accumulate in the head and around the amnioserosa and show very poor phagocytic activity despite their recruitment at sites of abundant apoptosis (E and F). Asterisks indicate the nucleus of each macrophage seen in these fields. (**G**) A chart summarizes the efficiency of phagocytosis of apoptotic corpses observed within each genotyped embryos assayed. Results shown are the mean P.I. ± SE; *n* is the total number of macrophages scored for each genotype. Dark blue, *w; In(2L)Cy/In(2L)Cy*; red, *w; In(2L)Cy/Df(2L)al*; yellow, *w; Df(2L)al/Df(2L)al*; and light blue, *w; W88/W88*.

role of this receptor in phagocytosis of apoptotic cells and in other macrophage functions in vivo.

To look at the *crq*-null phenotype, we used two overlapping deletions of the 21C region, *Df(2L)al* (*19*) and *Df(2L)TE99(Z)XW88* (*W88*) (*20*). The *Drosophila* genome project sequence indicates that *crq* is at position 21C4 between *expanded* (*ex*) (*21*) and *u-shaped* (*ush*) (*22*, *23*). *Df(2L)al* removes about 180 kb from the *aristaless* gene (*al*) (*19*) to *ush*, whereas *W88* uncovers about 100 kb from *ex* to *ush* (*20*). Homozygous embryos for either of these deficiencies can be distinguished by morphological defects (*19*, *22*). Both polymerase chain reaction (PCR) on single embryos (*24*) and CRQ immunostaining (*11*) confirmed that these homozygous embryos are *crq* null.

We assayed phagocytosis of apoptotic corpses in *Df(2L)al* and *W88* homozygous embryos with a double fluorescent immunolabeling for CRQ and peroxidasin, a hemocyte marker (*10*), and a nuclear dye, 7-amino actinomycin D (7-AAD) (*25*). Macrophages in wild-type embryos and embryos homozygous for the balancer chromosome were phagocytic for apoptotic corpses (Fig. 1, A and D) with a mean phagocytic index (P.I.) of 3.96 corpses per macrophage (Fig. 1G) (*26*). Although they accumulated at the site of cell death, macrophages within *Df(2L)al* and *W88* homozygous embryos remained very small and round (Fig. 1, B, C, E, and F), with P.I.s of 0.26 and 0.21, respectively (Fig. 1G).

In the absence of *crq* single mutants, we could not definitively conclude that the phenotype observed in *Df(2L)al* or *W88* homozygous embryos resulted solely from the deletion of *crq*. Therefore, we generated a UAS-crq transgene (*27*) and tested the ability of ubiquitously expressed CRQ to rescue the engulfment defect in *Df(2L)al* homozygous embryos, using a hsGal4 transgene to drive expression. In heat-shocked mutant embryos that carried both the hsGal4 and UAS-crq transgenes, macrophages showed substantial CRQ expression and phagocytic activity for apoptotic cells, with a P.I. of 2.20 (Fig. 2, C, F, and G). This indicates that *crq* is sufficient to rescue the phagocytosis defect in *Df(2L)al* homozygous embryos.

In serial sections of embryos that ubiquitously expressed CRQ, we observed that apoptotic corpses were not engulfed by cells other than macrophages (*28*, *29*). Thus, ectopic expression of CRQ is not sufficient to confer phagocytic ability on other cells in the embryo. This finding is in contrast with our previous observation that CRQ expression was sufficient to confer phagocytic activity on Cos7 cells (*13*). However, in *UAS-crq; hsGal4* embryos, CRQ was found at only low levels in cells other than macrophages, suggesting that CRQ might be unstable in other cells.

A human macrophage receptor, CD14, participates in both recognition and engulfment of pathogens as well as of apoptotic cells (*30*, *31*). We tested whether *crq* participates in phagocytosis of pathogens by *Drosophila* embryonic macrophages. We injected fluorescently labeled bacteria into living stage 11 wild-type and *W88* embryos and monitored their fate by confocal microscopy (*32*). Wild-type embryonic macrophages engulfed both Gram-negative (*Escherichia coli*) (Fig. 3, A to C) and Gram-positive bacteria (*Staphylococcus aureus*) (*28*). In *W88* homozygous embryos identified by their u-shaped phenotype (Fig. 3D), macrophages also engulfed bacteria (Fig. 3, D and E). Although these assays are not quantitative, we conclude that *crq* is specifically required for the phagocytosis of apoptotic corpses and is not essential for the engulfment of bacteria. *crq* is also not necessary for endocytosis of acetylated low density lipoproteins (LDLs) (*33*) (Fig. 3F) or for the production of the extracellular matrix components peroxidasin and MDP-1 (Fig. 1, B and C) (*28*).

The onset of CRQ expression corresponds to the time at which developmentally regulated apoptosis begins. We tested whether the presence of apoptotic cells might regulate CRQ expression by examining CRQ protein levels in embryos with altered amounts of apoptosis. In *Df(3L)H99* (*H99*) homozygous embryos, apoptosis does not occur, as a result of the deletion of the cell death regulators *reaper* (*rpr*), *grim*, and *head involution defective* (*hid*) (*34*–*36*). However, macrophages in *H99* homozygotes engulf corpses when apoptosis is induced by high levels of x-ray irradiation (*34*). When quantified by

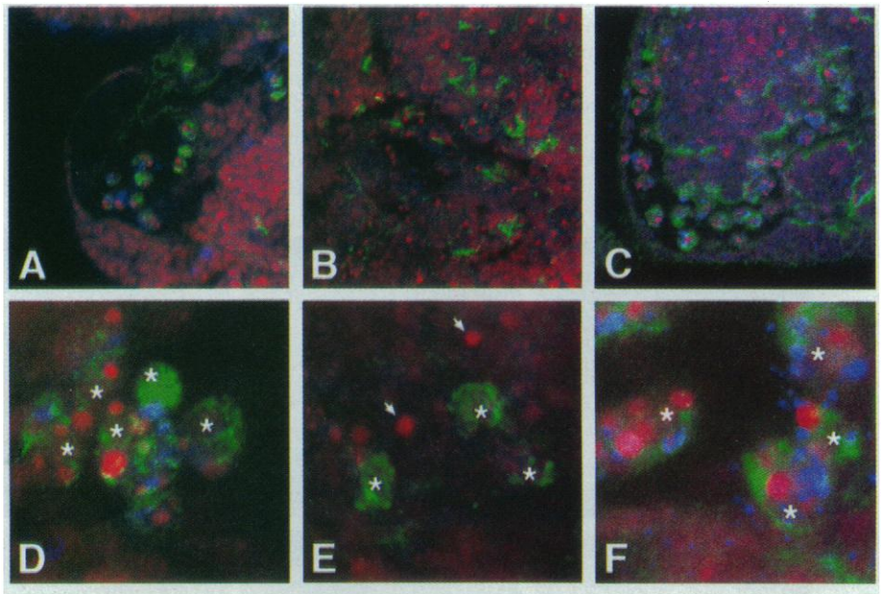

Fig. 2. Expression of a croquemort transgene reinstates the ability of macrophages in *Df(2L)al* homozygotes to recognize and engulf apoptotic corpses. A UAS-crq transgene was expressed under the control of a hsGal4 driver in all cells of *Df(2L)al* embryos, and phagocytosis of apoptotic corpses was assessed as in Fig. 1 (*25*). (**A** to **C**) Single focal planes. (**D** to **F**) Images are the sum of eight focal planes. (A) to (C) show a ×100 view of macrophages within the head of embryos with the following genotypes: (A) control: *w; CyO,S/CyO,S; hsGal4/+*; (B) homozygous mutant, as recognized by aberrant morphology: *w; Df(2L)al/Df(2L)al; hsGal4/hsGal4*; and (C) transgene rescue of homozygous mutant: *w, UAS-crq; Df(2L)al/Df(2L)al; hsGal4/+*. All embryos were heat-shocked for 1 hour at 39°C and assayed 2 hours later. (D) to (F) show high-magnification views (×400) of macrophages within the embryos shown in (A) to (C), respectively. Asterisks indicate the nucleus of each macrophage seen in these fields. Arrows indicate free apoptotic corpses. (**G**) A chart summarizes the efficiency of phagocytosis of apoptotic corpses observed within each category of embryos assayed. Results shown are the mean P.I. ± SE; *n* is the total number of macrophages scored for each genotype. Blue, *w; CyO,S/CyO,S; hsGal4/hsGal4*; red, *w; Df(2L)al/Df(2L)al; hsGal4/hsGal4*; and yellow, *w, UAS-crq; Df(2L)al/Df(2L)al; hsGal4/+*.

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confocal microscopy, CRQ expression was decreased by 74% in *H99* embryos (Fig. 4, B and E) as compared with wild-type embryos (Fig. 4, A and D) (*37*). Some hemocytes in these embryos do not express detectable levels of CRQ (Fig. 4H). However, after x-ray irradiation, apoptosis is induced in *H99* embryos (*34*), and CRQ expression increases (*28*). This suggests that *rpr*, *grim*, and *hid* themselves do not regulate CRQ expression but that the absence of apoptotic corpses results in CRQ down-regulation. MDP-1 expression is also down-regulated in *H99* embryos (*38*), suggesting that multiple macrophage functions might be activated in the presence of apoptotic cells.

We tested whether increased apoptosis resulted in increased CRQ expression by subjecting wild-type embryos to x-ray irradiation (*34*). In such embryos, giant macrophages were seen that had engulfed many apoptotic corpses. In these embryos, macrophages showed a 3.3-fold increase in CRQ expression as compared with wild-type embryos (Fig. 3, C and F) (*37*). CRQ expression was similarly up-regulated after treatment of *l(2)mbn* cells with ecdysone (*39*), which induces increased apoptosis and increases the phagocytic activity in these cells (*40*). Thus, signals generated by dying cells cause increased expression of CRQ, which could facilitate the clearance of the cell corpses. The expression of the related protein CD36 in human monocytes is also increased by binding to one of its ligands, oxidized LDL (*41*).

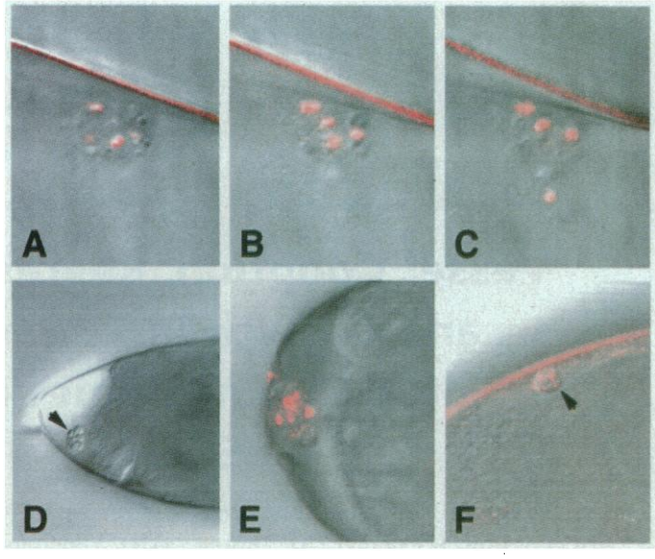

Fig. 3. Wild-type and *crq*-deficient *Drosophila* embryonic macrophages are competent to recognize and engulf bacteria and perform endocytosis. Stage 11 *yw* and *W88* homozygous embryos were injected with TRITC-labeled bacteria (*32*). The fate of the bacteria was monitored by confocal microscopy. (**A** to **C**) High-magnification images (×100) of a macrophage in a *yw* injected embryo taken at three successive focal planes. The overlay of Nomarski and fluorescent images shows a macrophage that has engulfed several labeled bacteria. (**D**) A Nomarski image of a magnified view (×40) of the head region of a *W88* embryo injected with TRITC-labeled bacteria. In this panel, a very large cell can be seen that has the typical morphology of a macrophage (arrowhead). (**E**) As seen at high magnification (×400), macrophages in this mutant can engulf bacteria. (**F**) A high-magnification image (×100) of a macrophage (arrowhead) in a *W88* embryo that had been injected with dioctadecyl tetramethyl indocarbocyanine–labeled acetylated LDL (DiI AcLDL) (*33*). Nomarski and fluorescent images of a single focal plane were merged so that the morphology of a macrophage that had taken up DiI AcLDL (red stain) can be seen.

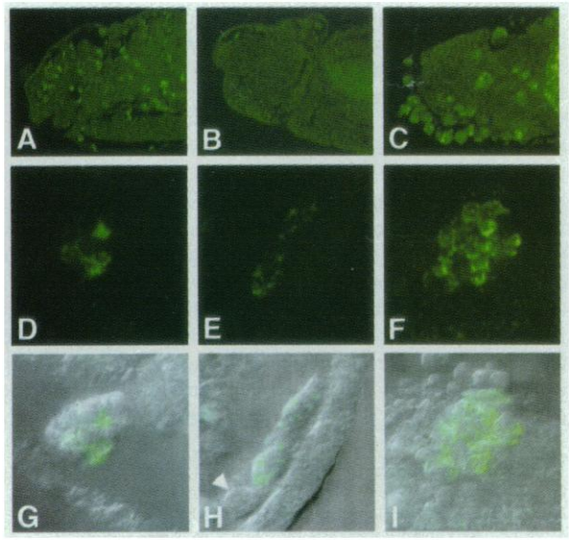

Fig. 4. *Croquemort* expression is regulated by the amount of apoptosis. Projected confocal images of a CRQ immunostaining in the head of a wild-type embryo (**A**, **D**, and **G**), an *H99* homozygous embryo (**B**, **E**, and **H**), and an irradiated wild-type embryo (*34*) (**C**, **F**, and **I**). (A) to (C) are at ×40; (D) to (I) are isolated macrophages at ×400. Images shown in (A) to (F) were taken with constant excitation and detection settings to show relative levels of CRQ staining. (G) to (I) show overlays of (D) to (F) with corresponding Nomarski images. CRQ expression is considerably down-regulated in *H99* homozygous embryos (B and E). In x-ray–irradiated wild-type embryos, the amount of apoptosis is considerably increased. Macrophages become greatly enlarged as they engulf numerous apoptotic corpses, and the level of CRQ expression in each macrophage is remarkably up-regulated (C and F). (E) and (H) show three small macrophages side by side, one of which does not appear to express CRQ (arrowhead). (D), (G), (F), and (I) show single macrophages.

This work characterizes a phagocytosis mutant in *Drosophila* and indicates that the CRQ protein is necessary, but probably not sufficient, for efficient phagocytosis of apoptotic cells in the embryo. Blocking studies on mammalian macrophages predicted a role for CD36 in the engulfment of apoptotic cells, and our in vivo data support this model. Because phagocytosis of apoptotic cells was not completely abolished in *crq*-deficient embryos (Fig. 1F), other receptors are probably involved in this process. Two other *Drosophila* macrophage receptors, the Scavenger Receptor dSR-C1 and Malvolio (*42*, *43*), may share overlapping functions with CRQ in the engulfment of apoptotic corpses. However, the rather low efficiency of the residual phagocytic activity in *crq*-deficient embryos implicates the CRQ pathway as a major participant in the phagocytosis of apoptotic cells. CRQ is not required for the phagocytosis of bacteria by embryonic macrophages, but because dSR-C1 and Malvolio are similar to molecules involved in mammalian immune responses (*42*, *43*), they may be specific pattern-recognition receptors for pathogens.

A genetic dissection of phagocytosis in *Drosophila* should further elucidate the phagocytic pathways for apoptotic corpses during development and for the engulfment of pathogens during an immune response. A greater understanding of the molecular mechanisms of both these processes in the fly, as well as of the macrophage responses they trigger, is likely to provide insights relevant to mammalian systems.

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25. CRQ immunostaining was used to genotype each embryo. Peroxidasin immunostaining detected all hemocytes [R. E. Nelson *et al.*, *EMBO J.* **13**, 3438 (1994)]. The nuclear dye 7-AAD labeled all DNA and allowed for the identification of apoptotic corpses. Unless otherwise specified, stage 11 to 16 embryos were fixed with standard procedures (*44*). Fixed devitellinized embryos were incubated in phosphate-buffered saline (PBS), 0.0125% saponin, 1% bovine serum albumin, and 4% normal goat serum (PSN) for 1 hour at room temperature and then incubated with the primary antibodies at a 1:1000 dilution in PSN overnight at 4°C. After several washes in PBS, the embryos were incubated for 1 hour at room temperature with the following secondary antibodies: fluorescein isothiocyanate–conjugated goat antibody to mouse and Cy5-conjugated goat antibody to rabbit (Jackson Immunoresearch) used at a 1:1000 dilution in PSN. Finally, embryos were washed three times in PBS for 20 min and subsequently incubated with 7-AAD (5 μg/ml) in PBS for 30 min. Embryos were quickly washed twice in PBS, mounted in Vectashield (Vector), and viewed by confocal microscopy (Leica TCS NT 4D).
26. The efficiency of engulfment was quantified by counting the number of engulfed corpses per macrophage in at least five fields of four embryos of each genotype. A P.I., that is, the mean number of engulfed corpses per macrophage, was calculated for each embryo, and the mean P.I. was derived for each genotype.
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Vessel Cooption, Regression, and Growth in Tumors Mediated by Angiopoietins and VEGF

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In contrast with the prevailing view that most tumors and metastases begin as avascular masses, evidence is presented here that a subset of tumors instead initially grows by coopting existing host vessels. This coopted host vasculature does not immediately undergo angiogenesis to support the tumor but instead regresses, leading to a secondarily avascular tumor and massive tumor cell loss. Ultimately, however, the remaining tumor is rescued by robust angiogenesis at the tumor margin. The expression patterns of the angiogenic antagonist angiopoietin-2 and of pro-angiogenic vascular endothelial growth factor (VEGF) suggest that these proteins may be critical regulators of this balance between vascular regression and growth.

It is widely accepted that most tumors and metastases originate as small avascular masses that belatedly induce the development of new blood vessels once they grow to a few millimeters in size (*1–3*). Initial avascular growth would be predicted for tumors that arise in epithelial structures that are separated from the underlying vasculature by a basement membrane and for experimental tumors that are implanted into avascular settings (such as the cornea pocket) or into a virtual space (such as the subcutaneum) (*2*, *3*). However, there is also evidence to suggest that tumors in more natural settings do not always originate avascularly, particularly when they arise within or metastasize to vascularized tissue (*4*). In such settings, tumor cells may coopt existing blood vessels (*4*). The interplay between this coopting of existing vessels and subsequent tumor-induced angiogenesis has not been extensively examined nor has the role of angiogenic factors in this process.

The pro-angiogenic vascular endothelial growth factors (VEGFs) and the angiopoietins are the only known growth factor families that are specific for the vascular endothelium because expression of their receptors is restricted to these cells (*5*, *6*). The angiopoietins include both receptor activators [angiopoietin-1 (Ang-1)] and receptor antagonists [angiopoietin-2

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